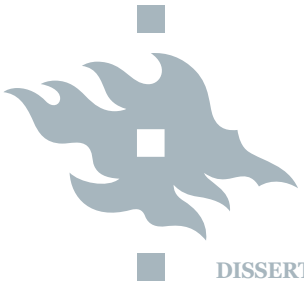

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

62/2017

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Cytotoxic and Apoptotic Effects of Selected Phenolic Compounds and Extracts from Edible Plants

DIVISION OF PHARMACEUTICAL BIOSCIENCES
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI

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CYTOTOXIC AND APOPTOTIC EFFECTS OF SELECTED PHENOLIC COMPOUNDS AND EXTRACTS FROM EDIBLE PLANTS

Tiina A. Lantto

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2, Viikki Infocentre on 10 November 2017 at 12 noon.

Helsinki 2017

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ISBN 978-951-51-3799-9 (Paperback)
ISBN 978-951-51-3800-2 (PDF)
ISSN 2342-3161 (Print)
ISSN 2342-317X (Online)
http://ethesis.helsinki.fi
Hansaprint 2017

To My Father

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Lantto TA**, Colucci M, Zavadova V, Hiltunen R and Raasmaja A (2009) Cytotoxicity of curcumin, resveratrol and plant extracts from basil, juniper, laurel and parsley in SH-SY5Y and CV1-P cells. Food Chemistry 117, 405-411.

II **Lantto TA**, Dorman HJD, Shikov AN, Pozharitskaya ON, Makarov VG, Tihonov VP, Hiltunen R and Raasmaja A (2009) Chemical composition, antioxidative activity and cell viability effects of a Siberian pine (*Pinus sibirica* Du Tour) extract. Food Chemistry 112, 936-943.

III Dorman HJD, **Lantto TA**, Raasmaja A and Hiltunen R (2011) Antioxidant, pro-oxidant and cytotoxic properties of parsley. Food and Function 2, 328-337.

IV **Lantto TA**, Laakso I, Dorman HJD, Mauriala T, Hiltunen R, Kõks S and Raasmaja A (2016) Cellular stress and p53-associated apoptosis by *Juniperus communis* L. berry extract treatment in the human SH-SY5Y neuroblastoma cells. Int J Mol Sci 17, 1113.

The publications are referred to in the text by the Roman numerals. In addition, some unpublished results are presented. The original papers are reprinted with the permission of the publishers.

CONTRIBUTION OF THE AUTHOR TO PUBLICATIONS I-IV

I The author is the primary author. The author designed the study in conjunction with supervisors, established cell models and assays, carried out the majority of the laboratory work, and analysed the data.

II The author is the co-author responsible for cell-based experiments. The author designed the study in conjunction with the other authors, planned and carried out laboratory work, and analysed the data.

III The author is the co-author responsible for cell-based experiments. The author designed the study in conjunction with the other authors, planned and carried out the laboratory work, and analysed the data.

IV The author is the primary author. The author designed the study in conjunction with the other authors, planned and carried out the laboratory work, and analysed the data.

The author is the primary author of all the unpublished data. The author designed the studies, planned and carried out the laboratory work, and analysed the data.

LIST OF ABBREVIATIONS

A375	Human melanoma cell line
Ac	Acetone
Ac-DEVD-AMC	Fluorogenic caspase 3 substrate; Acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin
AIF	Apoptosis inducing factor
AMC	7-amido-4-methylcoumarin
AMPK	Regulator of cellular energy homeostasis; AMP-activated protein kinase
ANOVA	Analysis of variance
AraC	Cytarabine / Arabinofuranosyl cytidine
ATM	Serine/threonine protein kinase
Aq	Aqua
ASC	Apoptosis-associated speck-like protein
Atg	Autophagy-related
Bak	Pro-apoptotic protein; Bcl-2 homologous antagonist/killer
Bax	Pro-apoptotic protein; Bcl-2 associated X
BCA	Bicinchoninic acid
Bcl-2	Anti-apoptotic protein; B-cell lymphoma 2 protein
Bcl-XL	Anti-apoptotic protein; B-cell lymphoma-extra large
BiP	Endoplasmic reticulum (ER) chaperone protein
BLASTN	Basic local alignment search tool for nucleotides
Ca^{2+}	Calcium-ion
CALM2	Calmodulin 2 (gene)
Caspase	Cysteine-dependent aspartate-directed protease
cDNA	Complementary deoxiribonucleic acid
cDNA RDA	Complementary deoxiribonucleic acid representational difference analysis
CO_2	Carbon dioxide
CV1-P	African green monkey fibroblast cell line
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxiribonucleic acid
DP1/2	First/second difference product
DPPH	Free radical; 2,2-diphenyl-1-picrylhydrazyl
ds	Double-stranded
DTT	Redox reagent; dithiothreitol

E. coli	Bacteria *Escherichia coli*
EDTA	Chelating agent; ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ER	Endoplasmic reticulum
EtOH	Ethanol
FADD	Fas-associated death domain
Fas / FasL	Death receptor Fas; Ligand of Fas receptor
H_2O	Water
HCl	Hydrochloride
HEPES	Buffering agent, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMPC	EMA Committee on Herbal Medicinal Products
HOAc	Acetic acid
HSPA5	Heat Shock Protein Family A (Hsp70) Member 5 (gene)
JNK	c-Jun N-terminal kinase
IAP	Inhibitor of apoptosis
IARC	The International Agency for Research on Cancer
IgG-HRP	Immunoglobulin G – horseradish peroxidase
LDH	Lactate dehydrogenase
MAC	Mitochondrial apoptosis-induced channel
Mcl-1	Anti-apoptotic protein; Bcl-2-like protein 3
Mdm2/4	Inhibitors of p53, E3 ubiquitin-protein ligases
MeOH	Methanol
$MgCl_2$	Magnesium dichloride
MilliQ H_2O	Ultrapure water
MOMP	Mitochondrial outer membrane permeabilisation
mTOR	Serine/threonine kinase
MTT	Tetrazolium dye; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$Na_2H_2PO_4$	Sodium dihydrogen phosphate
Na_2HPO_4	Sodium hydrogen phosphate
NCCD	Nomenclature Committee for Cell Death
NF-κB	Family of transcription factors
p21	Cell cycle inhibitor; transcriptional target of p53
p53	Transcription factor
p65	Transcription factor, member of NF-κB protein family
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PTM	Posttranslational mutation
RDA	Representational difference analysis
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxidative species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of a mean
SH-SY5Y	Human neuroblastoma cell line
SD	Standard deviation
STRING database	Search tool for the retrieval of interacting genes/proteins
SwissProt	Protein sequence database
TAE	Tris-acetate-EDTA buffer
TBP	TATA-binding protein, transcription factor
TE	Tris-EDTA buffer
TNFR / TNF	Death receptor TNF / Ligand of TNF receptor
TRAIL-R / TRAIL	Death receptor TRAIL / Ligand of TRAIL receptor
Triton X-100	Non-ionic surfactant; polyethylene glycol *tert*-octylphenyl ether
Tris-HCl	Buffer; trisaminomethane-hydrochloride
TBS	Tris-buffered saline
Tween 20	Nonionic detergent, polyoxyethylenesorbitan monolaurate
UniProtKD	The UniProt knowledgebase of functional information on proteins
UPR	Unfolded protein response
UV	Ultraviolet
XIAP	X-linked inhibitor of apoptosis protein
Ykt6	SNARE protein Ykt6

ABSTRACT

Plant phenolics and extracts are able to affect cell signalling associated with regulated cell-death mechanisms. Such mechanisms play a crucial role in the normal homeostasis of an organism, but inadequately functioning cell-death machinery is a component of the development of complex diseases, such as cancer, where cells divide in an uncontrolled manner. Apoptosis is the most studied regulated cell-death mechanism associated with cancer. One of the key triggers of and contributors to apoptotic cell death is a tumour suppressor p53. This protein is constantly produced, though it is activated only by several cellular stress responses, such as endoplasmic reticulum stress. The purpose of this study is to investigate the cytotoxic and apoptotic properties of three plant phenolics – curcumin, resveratrol and quercetin – and seven plant extracts – basil, juniper, laurel, lemon balm, parsley, and Siberian pine – in cancerous neuroblastoma and melanoma, and non-cancerous fibroblast cell models. The emphasis of the work is on plant extracts due to their claimed additive or synergistic effects on cellular mechanisms. The effects of different treatments are determined by two cell-viability tests, followed by Western blot assays of the amounts of p53, anti-apoptotic Bcl-2, and inflammatory p65. Apoptotic events are defined by the activity of caspase 3 and DNA fragmentation. Further testing to reveal a broader spectrum of effects is defined by the cDNA RDA method in order to investigate genes expressed differently in treated and in untreated cells. The results of this study support existing knowledge of the effects of single-plant phenolics, and reveal new mechanisms for the activity of plant extracts. Possible synergistic or additive effects of juniper plant extract on apoptosis through endoplasmic reticulum stress are observed. Plant phenolics and extracts may provide a unique pool of drug candidates for the prevention or treatment of cancer. The use of plant extracts as drug candidates is especially interesting due to the possible synergistic or additive effects they achieve at low concentrations.

1 INTRODUCTION

Edible plants are rich sources of phenolic compounds in human diets and have been shown to possess potential anti-cancer properties that regulate cell-death pathways inhibiting the development and progress of cancer (Anantharaju et al. 2016). Plant phenolics are the main secondary metabolites of plants, defending them against external threats such as herbivores and microbes, and acting as signalling molecules to attract fruit dispersers (Wink 2016). They are strong antioxidants and are also capable of binding receptors and modulating signalling proteins of other organisms, such as humans (Williams et al. 1989). The majority of anti-cancer drugs on the market are plant-derived or are synthetic derivatives of them (Pan 2010, Newman and Cragg 2016).

Modern Western medicine has overcome several lethal diseases with the classical pharmacological approach of using a single molecule for a single target; however, several complex diseases, such as cancer, still lack definitive and cost-effective treatment and prevention strategies. Today, there are 8.2 million cancer-related deaths reported worldwide annually; the annual 14.1 million new cancer diagnoses are predicted to rise to 20 million worldwide by 2025 (IARC 2014). The costs involved will rise accordingly, from the annual €270 billion recorded in 2010. Over half of the total costs are used for medical treatments (Bloom et al. 2011, IARC 2016). It has been evaluated that over half of drug treatments today have no effect, the effect is insufficient, or the treatment has serious adverse effects (Juntti-Patinen and Neuvonen 2002; Niemi 2010; Spear, Heath-Chiozzi and Huff 2001). The need for both basic and applied research to search for new strategies to prevent and treat cancer is crucial in order to decrease immense humane suffering and the economic burden caused by the disease.

A combination approach, using a mixture of drugs, has been acknowledged in drug development processes in past years (Newman and Cragg 2016, Constantino and Barlocco 2012, Narain 2016). The strategy

of medicating with mixtures of compounds or plant extracts is not new. Traditional medicinal systems, such as traditional Chinese medicine and Indian Ayurveda, have utilised the potential synergistic effects of herbal medicines for hundreds to thousands of years (Bhandaria et al. 2015, Liu et al. 2015). The same plants could be utilised in combination with chemotherapy today. The benefits of mixtures of low-affinity molecules administered in low doses are the avoidance of cancer cells developing resistance to drugs, and the lack of adverse side-effects common to conventional chemotherapeutic agents (Efferth 2010a/b).

Drug development in the 21st century has focused on mapping the key signalling molecules of cell-death pathways, including apoptosis (Persidis 1998). Potential targets to prevent or treat cancer can be found in such pathways, which play key roles in the initiation and progression of cancer. One of the most studied signalling proteins associated with cell death is the tumour-suppressor protein p53. It is an interesting marker protein due to its extensive role in detecting cellular stress and mediating cell-cycle arrest and cell death. In this study, selected plant phenolics and plant extracts from edible plants are investigated for their cytotoxicity, their effect on p53, and for other apoptotic cancer-related mechanisms.

2 LITERATURE REVIEW

2.1 CELL DEATH

The death of cells can be either accidental or regulated. Regulated cell death is genetically strictly operated and is an essential biological phenomenon for multicellular organisms (Galluzzi et al. 2015). In healthy cells, it is involved in several biological processes, such as development, homeostasis, embryogenesis, maturation of the immune system, and protecting an organism from microbial attacks. The activity of regulated cell death is not limited to functions in healthy cells – it plays a significant role in several pathological conditions, such as cancer, chronic inflammation, and neurodegenerative diseases. In such conditions, the mechanisms of death are impaired by increasing or decreasing cells' ability to self-destruct. Accidental or necrotic cell death is not controlled, and it occurs as a result of physical (e.g. temperature), chemical (e.g. pH), or mechanical (e.g. shearing) stimuli.

Healthy cells do not die spontaneously; the difference between life and death depends on simultaneous pro-death and pro-survival signals and strictly regulated signalling pathways (Lockshin 2016, Flusberg and Sorger 2015). Due to the biological importance of cell death for both physiological and pathological conditions, it is not surprising that there are several conservative, overlapping, and complementary mechanisms involved in the removal of unwanted cells in multicellular organisms. Apoptosis was the first cell death mechanism discovered, and is currently the most well-defined cell-death type, garnering over 345,000 hits in a Pubmed search on 12 June 2017. Other cell-death mechanisms that have been discovered include, for example, autophagic cell death, necroptosis, and pyroptosis (Kroemer et al. 2005, Cookson and Brennan 2001).

Different types of cell death are conventionally distinguished by their biochemical and morphological features, however, recently the

Nomenclature Committee for Cell Death (NCCD) has proposed favouring biochemical characteristics (Galluzzi et al. 2012). Although inflammation is conventionally related to accidental or necrotic cell death, some regulated cell-death types also induce inflammation. Apoptosis and autophagic cell death do not initiate inflammation due to membrane-based mechanisms that protect neighbouring cells and other surroundings from harmful cell contents. The biochemistry, the existence of pro-inflammatory properties, and activation of selected cell-death types are summarised in Table 1.

Table 1. Comparison of cell-death mechanisms (Galluzzi et al. 2012, Galluzzi et al. 2015)

CELL-DEATH TYPE	PRO-INFLAMMATORY	BIOCHEMICAL CHARACTERISTICS	ACTIVATION
Apoptosis	No	Activation of caspases 8, 9 and 3; DNA fragmentation	Development and homeostasis, ER stress
Autophagic cell death	No	MAP1LC3 lipidation, SQSTM1 degradation	Starvation, ER stress, toxic chemicals
Regulated necrosis / Necroptosis	Yes	Death-receptor signalling, RIP1 and RIP3	Alkylating DNA damage, ex-citotoxins, ligation of death receptors
Pyroptosis	Yes	Activation of caspases 1 and 7, secretion of IL-1b and IL-18	Microbial infection, heart attack, cancer

2.2 MECHANISMS OF CELL DEATH

2.2.1 Apoptosis

Apoptosis is the form of programmed cell death involved in development and tissue homeostasis. It was first described by histologists and developmental biologists in the middle of 20th century and named by

Kerr, Currie and Wyllie (1972). Apoptosis is triggered by, for example, DNA damage, endoplasmic reticulum (ER) stress, oxidative stress, heat shock, viral infections, hypoxia, or xenobiotics such as chemotherapeutic agents, environmental pollutants and pesticides (Galluzzi et al. 2008; Orrenius, Nicotera and Zhivotovsky 2011).

During development and homeostasis, it is not well understood how the decision of death is defined, but the damaged cells die if repair of detected malfunctions is not possible. Depending on the trigger of death, apoptosis is activated by an intrinsic/intracellular or extrinsic/extracellular pathway. The main molecular event of the intrinsic pathway is the permeabilisation of the mitochondrial outer membrane (MOMP), whereas death receptors on cell membrane activate the extrinsic pathway. The cleavage of cellular contents by cysteine proteases called caspases is involved in both pathways. Extrinsic and intrinsic apoptosis and caspases are introduced in greater detail in the chapters that follow, and the overview of both pathways is introduced in Figure 1.

Morphological characteristics of apoptosis include DNA fragmentation and condensation of chromatin and cytoplasm. Shrunken cells are fragmented into membrane-bound apoptotic bodies which are then disposed of by dedicated phagocytes such as macrophages and immature dendritic cells, or by non-dedicated neighbouring cells such as fibroblasts and epithelial cells (Aderem and Underhill 1999; Kerr, Wyllie and Currie 1972; Monks et al. 2008; Wyllie 1980). Apoptotic cells in the early stage of death are recognised by macrophages by the translocation of phophatidylserine to the outer cell membrane (Hengartner 2001).

Apoptosis has the central role in cancer development. It is impaired in cancer cells, allowing aberrant cells to proliferate and to progress to form tumours. Due to the complexity of apoptotic signalling, dysfunctions can originate from several impaired proteins and pathways, and vary between cancer types and even between cells in the same tumour (Flusberg and Sorger 2015). According to Hassan and co-workers (2014), the main mechanisms and proteins impaired in apoptosis include the

elevated expression of anti-apoptotic proteins such as B-cell lymphoma protein 2 (Bcl-2) and Bcl-XL; expression of caspase 8 negative regulator c-FLIP, which is elevated, and is accompanied by the constitutive activation of its transcription factor nuclear factor-κB (NF-κB); and elevated expression of inhibitors of apoptosis (IAPs).

2.2.1.1 Intrinsic apoptosis

The MOMP is a critical step in the activation of intrinsic apoptosis. This process enables the release of pro-apoptotic proteins such as cytochrome c, Apaf-1 (apoptosis activating factor 1), endoG (endonuclease G) and Smac/Diablo (second mitochondria-derived activator of caspases / direct IAP-binding protein with low PI) from the intermembranous space of mitochondria (Montero et al. 2015). A large protein complex, apoptosome, is formed in the cytosol by cytochrome c, Apaf-1 and procaspase 9. Apoptosome activates initiator caspase 9, which further activates executer caspase 3, which is responsible for the final events of apoptosis.

MOMP is controlled by evolutionarily conserved pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family (Certo et al. 2006, Chipuk et al. 2010). Such proteins include effectors, activators, inhibitors, and sensitisers, and they all contain up to four Bcl-2 homology domains (BH1-4). Oligomerisation of effectors Bax and Bak on the outer membrane of mitochondria form mitochondrial apoptosis-induced channels (MACs) through the outer membrane of mitochondria. Bax and Bak can also cause MOMP without the formation of MAC through release of Ca^{2+} and its major regulator, calmodulin (Berchtold and Villalobo 2014). Effectors are activated by the BH3-only proteins Bid and Bim (Sarosiek et al. 2013). Anti-apoptotic Bcl-2, Bcl-XL, and Mcl1 inhibit both effector and activator proteins by binding (Certo et al. 2006) directly to Bax and Bak, or indirectly to Bid or Bim (Green and Kroemer 2004, Youle and Strasser 2008). The sensitisers Bad, Bmf, Noxa, and Hrk induce MOMP indirectly by inhibiting anti-apoptotic Bcl-2-family proteins. Intrinsic apoptosis is the most

common response of cancer cells to chemotherapeutic agents, and drugs inhibiting Bcl-2-family proteins are the latest advance in the treatment of cancer (Montero 2015, Ashkenazi et al. 2017).

2.2.1.2 Extrinsic apoptosis

Death receptors mediate extrinsic apoptosis by means of the formation of a death-inducing signalling complex (DISC). In the DISC, intracellular death domains of receptors interact with Fas-associated death domains (FADD) and activate initiator caspase 8 (Dickens et al. 2012) which mediates the caspase cascade by directly activating the effector caspase 3. If the interaction between caspases 3 and 8 is blocked, extrinsic apoptosis can be transmitted via MOMP. Caspase 8 then activates Bid, followed by the activation of Bak, which triggers MOMP and the formation of apoptosome (Sarosiek et al. 2013) or release of Smac/Diablo and IAPs which block the X-linked IAP (XIAP) (Galluzzi et al. 2015). These key events and agents in receptor-mediated apoptosis are not the full story in the complex signalling of extrinsic apoptosis where pro-apoptotic and anti-apoptotic signals act in parallel and are steered by IAPs.

Extrinsic apoptosis is triggered by the extracellular death ligands TNF, FAS and TRAIL which bind with cysteine-rich extracellular domains of death receptors TNFR-1, Fas and TRAIL-R1,2. Localisation of death receptors strengthens pro-apoptotic signals (Flusberg and Sorger 2015). Death receptors are able to activate caspase 8 and 3 within seconds, and apoptosis in hours (Ashkenazi and Dixit 1998). Despite their name, the death receptors are involved in activities other than stress-related pro-apoptotic signalling, including differentiation, proliferation, chemokine production, inflammation and the promotion of tumours. Some of these non-cytotoxic events are mediated via NF-κB and mitogen-activated protein kinase (MAPK) pathways. The role of NF-κB is linked to cell survival triggered by TNF-receptors (Guicciardi and Gores 2009).

2.2.1.3 Caspases

Caspases (intracellular cysteine-aspartic proteases) are the key agents of different types of cell death, including apoptosis, pyroptosis, anoikis, and cornification. Autophagic cell death, necroptosis, and mitotic catastrophe are independent of caspase activity (Galluzzi et al. 2012). Caspases are grouped as initiators (caspases 2, 8, 9, and 10) and effectors (caspases 3, 6, and 7) according to their specific function. They are produced as inactive zymogens, and they gain their catalytic activity of cleaving peptide bonds through molecular modifications. Initiator caspases are produced as procaspase monomers, and they are activated by dimerisation and interchain cleavage. Caspases act as cascades which activate one another. Initiators activate effector caspases 3, 6, and 7 which are produced as inactive dimers by the cleavage of interchains between sub-units. Caspases are not only crucial in cell death but also in inflammation, cytokine maturation, and differentiation. For example, caspases 1, 4, 5, and 12 have found to play a role in inflammatory processes (Man and Kanneganti 2016).

2.2.1.4 Endoplasmic reticulum (ER) stress

Prolonged ER stress can induce cell death through autophagic cell death or apoptosis. It is activated by detected protein accumulation or misfolding in response to chemotherapeutic agents, environmental pollutants or physiological conditions such as disturbed redox and calcium status, and deprivation in nutrients. Pathological conditions have also been shown to induce ER stress via impaired cellular homeostasis. Endoplasmic reticulum stress activates several signalling pathways called unfolded protein responses (UPR). One of the activated proteins is the transcription factor NF-κB. During ER stress, NF-κB is activated in response to elevated calcium release and the presence of reactive oxygen species (ROS). It has been shown to stimulate p53 expression, linking cell survival and death to ER stress (Lin et al. 2012). This stress is a potential target for pharmaceutical interference.

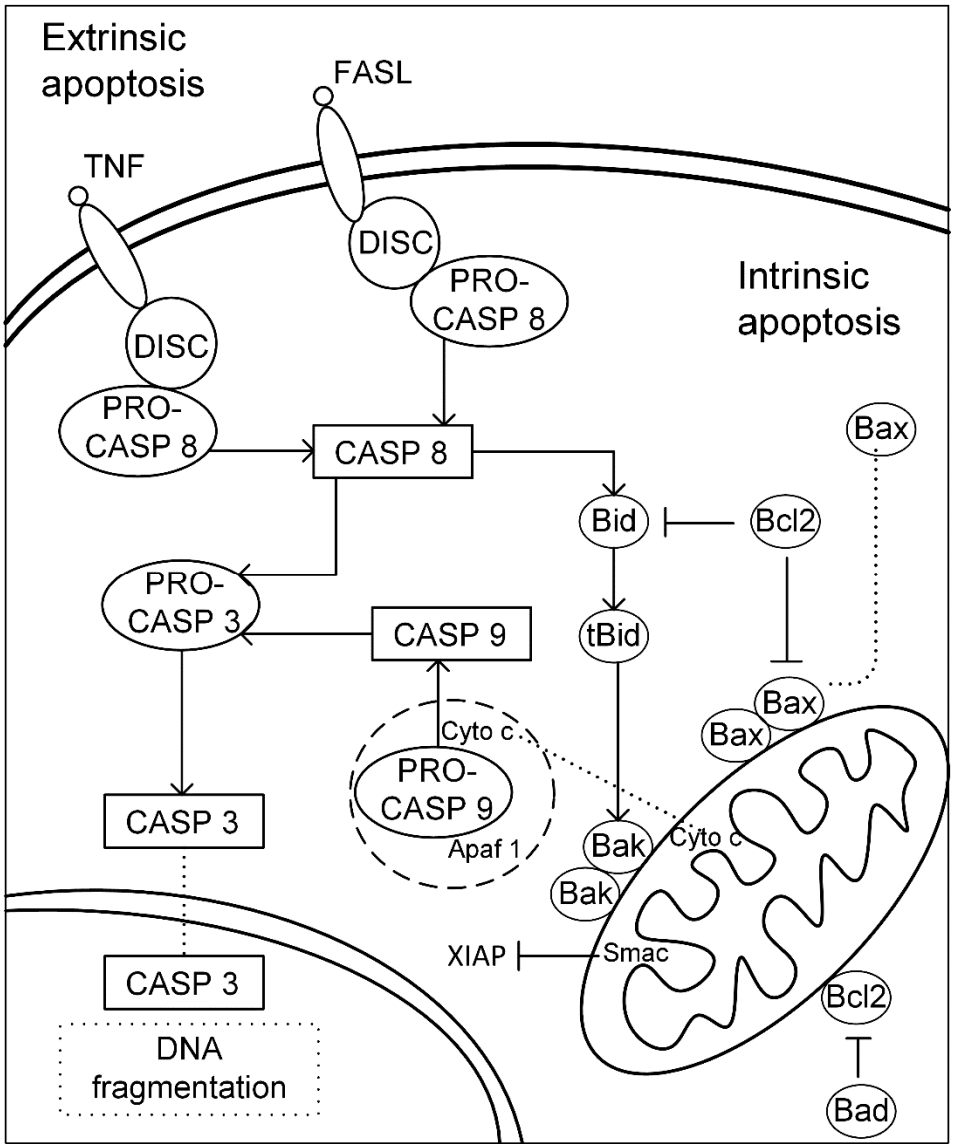

Figure 1. Simple overview of the intrinsic and extrinsic apoptotic pathways.

2.2.2 Autophagic cell death

Autophagy is a cytoprotective and evolutionarily conserved mechanism for removing misfolded proteins and cell organelles, and is closely linked to the cell-death processes. It controls physiological homeostasis by means of both general and selective mechanisms. General autophagy recovers energy for cells suffering starvation by delivering cytosolic material to lysosomes for degradation, while selective autophagy acts similarly in cells with damaged and/or unwanted organelles (Green and Levine 2014). In addition to starvation, other cellular stress conditions, such as hypoxia, mitochondrial damage, pathogens and toxic chemicals, trigger autophagy. Diseases and conditions related to the malfunction of autophagy include cancer, aging, and inflammation (Green and Levine 2014, Liu and Levine 2015).

In intracellular events of autophagy, cytosolic material and organelles are transported to lysosomes in double-membrane structures called autophagosomes. The fusion of autophagosomes and lysosomes is visible as vacuolisation which is the main morphological feature of autophagic cell death. To confirm the involvement of autophagy in cell death requires evidence of the activation of autophagy genes such as LC3B-II which associates with the autophagosome and induces its elongation (Fulda 2012, Lindqvist et al. 2014). Other biochemical identifiers for autophagic cell death are the lipidation of MAP1LC3 and the degradation of SQSTM1 (Galluzzi et al. 2012).

Apoptosis and autophagic cell death are closely linked, and apoptosis can be triggered by autophagy (Lindqvist et al. 2014). Whereas apoptosis is the mechanism to demolish the entire cell, autophagy is able to dispose of only parts of the cell. Interaction between autophagy and apoptosis is mediated through proteins common to both mechanisms. For example, the starvation-induced autophagy can be inhibited by the anti-apoptotic proteins Bcl2 and Bcl-XL (Wirth, Joachim and Tooze 2013). However,

Lindqvist and co-workers (2014) demonstrated that their role in autophagy is indirect, by inhibiting apoptosis via Bax and Bak.

Autophagy has a dual role in cancer. Its cytoprotective activity may promote tumour progression but when apoptosis is defunctional as a result of lack of pro-apoptotic proteins, autophagic cell death may be responsible of their death (Buytaert et al. 2006). Endoplasmic reticulum stress can induce both autophagy and apoptosis after detected disturbances in ER calcium homeostasis or function. Differences in response have been found between cancerous and normal tissues. For example, in colon and prostate cancer cells, the induction of autophagy promotes cell survival, while in non-cancerous human colon cells and murine fibroblasts, ER-induced autophagy has been found to trigger apoptosis (Nikoletopoulou et al. 2013).

2.2.3 Other mechanisms of cell death

2.2.3.1 Necrosis, regulated necrosis and necroptosis

The term necrosis has conventionally been considered a synonym for the accidental type of cell death. In certain conditions, such as alkylating DNA damage and ligating death receptors, necrotic cell death can be molecularly regulated (Zong et al. 2004, Zhang et al. 2009). Necroptosis is triggered by mitochondrial ROS or pro-inflammatory cytokine TNF-α. Increased levels of ROS induce necrotic cell death by damaging DNA, proteins, and lipids resulting in the pro-inflammatory death of cells (Festjens, Berghe and Vandenabeele 2006). Autophosphorylation of RIP1 mediates the necroptosis, activating RIP3 and forming a necrotic signalling-complex necrosome (Zhang et al. 2016). After phosphorylation, oligomerisation and translocation of mixed lineage kinase domain-like protein on the plasma membrane execute necroptosis by means of the necrosome. The term necroptosis is often used as a synonym for regulated necrosis even though it was introduced to describe cell death triggered by TNFR1 ligation. Necroptosis is involved in pathological

processes such as viral and bacterial infections, and inflammatory disorders induced by injuries (Chan, Luz and Moriwaki 2014).

2.2.3.2 Pyroptosis

Pyroptosis was discovered and named by Cookson and Brennan (2001) in the course of studies on microbial infections. Stimuli other than bacterial or viral infection that trigger pyroptosis include heart attacks, cancer, and strokes. Pyroptosis is the inflammatory mechanism of cell death that results in cell lysis, release of cytokines and inflammation (Kepp et al. 2010). Morphological features of pyroptosis, such as cytoplasmic swelling and DNA fragmentation, are similar to those of apoptosis and necrosis (Kepp et al. 2010). Pyroptosis is dependent on caspase activation; however, whereas caspases 3, 8 and 9 are activated in apoptosis, caspases 1 and 7 are involved in pyroptosis. Pyroptotic activation of caspase 1 occurs in an inflammasome, which is a large multiprotein complex, or a pyroptosome, a large supramolecular assembly of apoptosis-associated speck protein (ASC)BSA containing a caspase activation and recruitment domain (Fernandes-Alnemri et al. 2007). Caspase 1 induces the proteolytic maturation and release of pro-inflammatory cytokines IL-1β and IL-18.

2.3 P53

The tumour suppressor protein p53 senses cellular stress triggered by, for example, DNA damage, ribosomal and ER stress, ROS, chemotherapeutic agents, oncogene activation, aberrant growth signals and mitotic catastrophe (Loughery and Meek 2013; Vogelstein, Lane and Levine 2000). As a response to stress, p53 induces cell survival or death by its transcriptional and non-transcriptional actions (Moll et al. 2005). Since p53 was discovered in 1979, intensive research on this ‘guardian of the genome’ (Lane 1992) has produced over 86,000 publications according to the results of a Pubmed search on 12 June 2017. The enormous interest

in p53 arises from its involvement in several signalling pathways and in pathological conditions such as cancer (Junttila and Evan 2009).

The p53 protein is the most regularly mutated protein in human cancers (Hainaut and Hollstein 2000). Almost every human tumour involves the malfunction of p53 or related pathways, and in half of them p53 is mutated (Muller and Vousden 2013). Both somatic and germline mutations have been reported. Typical somatic mutations are missense, nonsense, and frameshift mutations. Missense mutations are the most prevalent, comprising more than 70% of all reported p53 mutations (IARC 2014). While somatic mutations are often reported for colorectal, neck, and head tumours, germline mutations are common for breast, soft tissue, brain, and adrenal gland tumours. About 97% of p53 mutations alter its DNA-binding domain as a result of impaired transcriptional activity (Olivier et al. 2002), leading to more aggressive cancer types, resistance to therapies, and the occurrence of oncogenic functions in p53 (Goldstein et al. 2011, Walerych et al. 2012, Wattel et al. 1994).

2.3.1 Functions of p53

The functions of p53 are primarily mediated by its transcriptional activity. It acts on over one hundred transcription target genes acting in apoptosis, cell-cycle arrest, metabolism, autophagy, and tumorigenesis (Riley et al. 2008; Bieging, Spano Mello and Attardi 2014). Other functions of 53 are located on the outer membrane of mitochondria and in cytosol, promoting pro-apoptotic and anti-apoptotic proteins though direct protein-protein interactions (Bieging, Spano Mello and Attardi 2014). Depending on the cellular stress detected, p53 induces the repair or elimination of cells by activating cell-survival and cell-death pathways, respectively. When malfunctions cannot be repaired, p53 promotes regulated cell-death mechanisms. In Figure 2, the stress responses and related mechanisms of p53, as detected in neuroblastoma cells following juniper berry-extract treatment in neuroblastoma cells, are introduced.

Apoptotic transcriptional targets of p53 include Apaf1, Bax, Fas, Noxa and Puma; the Bcl-2-family proteins Bax, Bak, Bcl-2 and Bcl-XL are regulated by direct p53 interactions in the cytosol (Moll et al. 2005). Thus, p53 has a dual role in Bax regulation. The p53-Bax interaction triggers conformational change in Bax, triggering its oligomerisation on the outer membrane of mitochondria as well as MOMP activity (Chipuk et al. 2004). The same outcome for Bak is achieved through the disruption of Bak-Mcl1 interaction by p53. Bak is the principal cytosolic target of p53 (Leu et al. 2004). In autophagy, p53 possesses the dual role of inducing or inhibiting the process (Tasdemir et al. 2008). As mentioned above, apoptosis and autophagy share their regulatory proteins and are closely linked to each other. Dram1 and Puma are transcription targets of p53 and they are both involved in apoptosis and autophagy (Bieging, Spano Mello and Attardi 2014; Crighton et al. 2006). Other autophagy-related transcriptional targets include Atg-proteins and Lkb1.

The cell survival functions of p53 include transient cell-cycle arrest, permanent cell-cycle arrest (senescence), autophagy, and metabolic programming (Zhang and Lozano 2016). The transcriptional targets of p53 in cell-cycle arrest and DNA repair include p21, 14-3-3, Btg2, and GADD45 (Riley et al. 2008; Bieging, Spano Mello and Attardi 2014). The principal pathway of cell-cycle arrest triggered by DNA damage is the inhibition of cell-cycle mediators such as Cdk1 and Cdk4 by p21 in the G1 and G2 phases of the cycle (Bunz et al. 1998, He et al. 2005).

Favourable metabolic alterations of cancer cells, including increased glucose uptake, fatty-acid synthesis, glutaminolysis, and mitochondrial biosynthesis are suppressed by p53 via the Lkb1/MAPK pathway (Phan, Yeung and Lee 2014). The mechanism for p53 to suppress such events by means of its transcriptional activity is the down-regulation of the glucose transporters Glut1 and Glut4, and the suppression of Glut3 by blocking its transcription factor NFκB (Kawauchi et al. 2008).

It is still unclear how a distinction is made between the need for repair and the need for elimination. It has been suggested that the higher

affinity of p53 for pro-survival genes compared to pro-apoptotic genes determines the fate of cells. On the other hand, Kracikova et al. (2013) demonstrate the importance of a threshold mechanism where pro-arrest and pro-apoptotic genes are activated proportionally. Furthermore, it has been suggested that the localisation of p53 affects the destiny of cells (Moll et al. 2005).

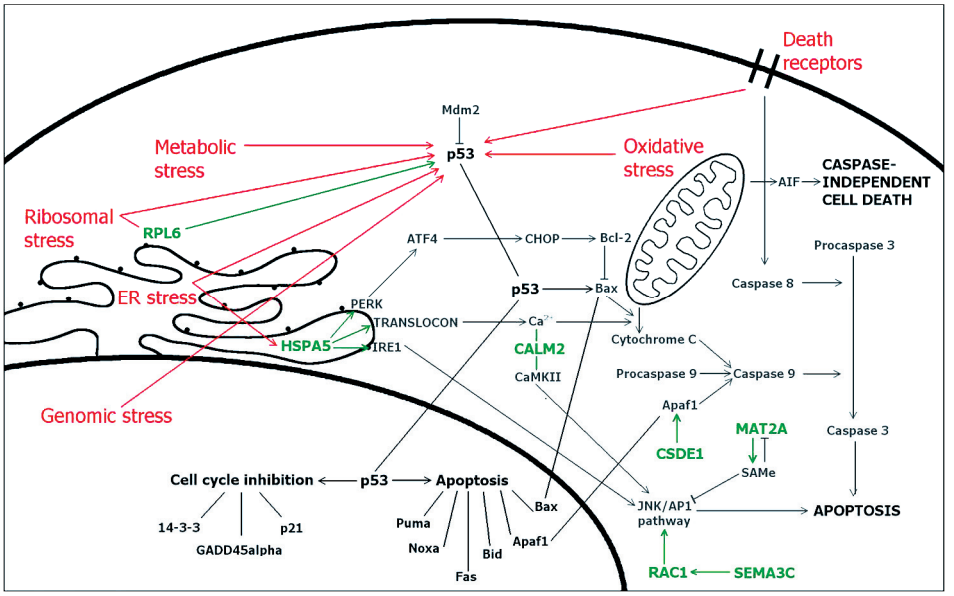

Figure 2. The role of p53 in selected stress reactions. Proteins marked in green were differentially expressed after juniper berry treatment in our studies (Lantto et al. 2016).

2.3.2 Regulation of p53

In healthy cells, p53 is constantly produced but it has extremely short cytosolic half-life, from 5 to 30 seconds (Davidoff et al. 1991; Rogel et al. 1985). Its intracellular quantity and activity is kept low by post-translational modifications (PTMs) that include ubiquitination, phosphorylation, acetylation, sumoylation, methylation, and neddylation. In fact, p53 is the protein richest in PTM sites and modificators (Meek and Anderson 2009). One example of p53 modification is the phosphorylation of Ser15 by the AMP-activated

protein kinase (AMPK) pathway, which results in cell-cycle arrest after stress signals consequent to DNA damage. Although numerous studies describe detailed PTMs for p53, their functional involvements are often controversial (Meek and Anderson 2009).

Mdm2 and Mdm4 are the main cytosolic inactivators of p53. Inactivation occurs by ubiquitinating it for proteosomal degradation (Kubbutat, Jones and Vousden 1997; Wade, Li and Wahl 2013). Mdm2 and p53 engage in an auto-regulatory feedback loop initiated by p53 to promote the expression of Mdm2, which in turn inhibits p53 by degrading it. Cellular stress signals trigger the breaking of the hydrophobic p53-Mdm2 interactions. This allows stabilised and activated p53 to move to the nucleus, cytosol or to the outer membrane of mitochondria. The importance of this mechanism of the inactivation of p53 in pathological conditions is demonstrated by the observation that both Mdm2 and Mdm4 are found to be overexpressed in many tumours (Toledo and Wahl 2006).

2.4 CANCER

Cancer is a heterogeneous disease that develops through the transformation of healthy cells to rapidly and uncontrollably dividing cancer cells as a result of, for example, environmental pollutants, gene oncogenes, or viruses. Chronic inflammation is also closely involved in the development of cancer, promoting tumorigenesis by means of several mechanisms (Pan, Lai and Ho 2010). Cancer is conventionally treated through surgery, chemotherapy, radiotherapy, and biotherapy. The slow development and progression of cancer makes prevention of the disease an important treatment approach (Valle, Tramalloni and Bragazzi 2015).

If cells detect cancerous alterations but their reparation and elimination processes are impaired, they are not able to commit self-destruct. Such impaired mechanisms may provide an early-stage target for chemotherapy. Chemotherapeutic agents are able to trigger cell-death

mechanisms such as apoptosis and autophagic cell death (Tasdemir et al. 2008). For example, doxorubicin induces p53-mediated apoptosis followed by caspase 3 activity and DNA fragmentation in tumour cells (Wang et al. 2004). This mechanism is accompanied by the synthesis of ceramide following the activation of transcription factor CREB3L1, which induces the expression of p21 (Denard, Lee and Ye 2012). Bortezomib is used to treat haematological malignancies by means of proteasome inhibition and apoptosis. It has been found to upregulate Noxa, Mcl-1 and HSP70, induce cleavage of LC3 and autophagy, the accumulation of ROS, and the release of AIF and cytochrome c, followed by the activation of caspases 9 and 3 (Selimovic et al. 2013). The greatest challenges with such drugs that assist in killing cancerous cells are their toxicity towards healthy cells, the development of resistance in cells, and the complex nature of tumours containing several mutations that affect their reactivity to drugs (Fojo 2008). For example, the use of doxorubicin is limited due to its severe cardiotoxicity mediated by calcium-related autophagy and the inhibition of rapamycin (mTOR) signalling (Park et al. 2016). Resistance to bortezomib develops by means of up-regulation of proteasome sub-units, alterations in gene and protein expression, the survival of cells and anti-apoptotic pathways, and multi-drug resistance (Lu and Wang 2013). New approaches with different mechanisms of action or more selective toxicity against cancerous cells are needed to minimise such adverse effects.

Combination therapy is the strategy of beating cancer by targeting multiple signalling pathways simultaneously with several drug molecules (Lehar et al. 2009). By covering a broad spectrum of molecular targets, the administration of multiple drugs may enable synergistic or additive effects which allow lower treatment concentrations with reduced toxicity (Parhi, Mohanty and Sahoo 2012). Other advantage of combination therapy is possible avoidance of the multi-drug resistance commonly developed when using single drug molecules. Plant phenolics and extracts may provide such combinations for use in chemotherapy and chemoprevention.

2.5 PLANT PHENOLICS AND EXTRACTS

Plant phenolics are the most abundant secondary metabolites produced by plants to protect them from stress and to attract pollinators. They can be found in herbs, fruits, vegetables, cereals, and beverages such as tea and red wine. Functions of plant phenolics include protection from microbes, fungi, insects, herbivores, free radicals generated during the photosynthetic process and by UV radiation, and chelation of toxic heavy metals. Plant phenolics are responsible for the plant colours that attract pollinators (Stevenson and Hurst 2007). More than 8,000 plant phenolics have been identified, including flavonoids, simple phenols, stilbenes, curcuminoids, lignans, lignins, and tannins. More than half of the identified structures are flavonoids (Tsao 2010). Plant phenolics contain one or more aromatic benzene rings with one or more hydroxyl groups. Based on the number of aromatic rings, they can be classified as either simple or complex plant phenolics. Simple plant phenolics with one aromatic ring include benzoic and cinnamic-acid derivatives; complex phenols with two or more aromatic rings include flavonoids, tannins, and stilbenes. Flavonoids can be further classified into sub-categories such as flavanones, flavones, flavans, and anthocyanines (Anantharaju et al. 2016). Figure 3 depicts selected phenolic classes and compounds relevant to this study. The abundance of phenols in different plants varies depending on the phenolic classes: stilbenes are produced in only a few plant species, but flavonoids are common to all plants (Flamini et al. 2013). Plant phenolic content varies according to seasonal changes and growing conditions (Tavares et al. 2013).

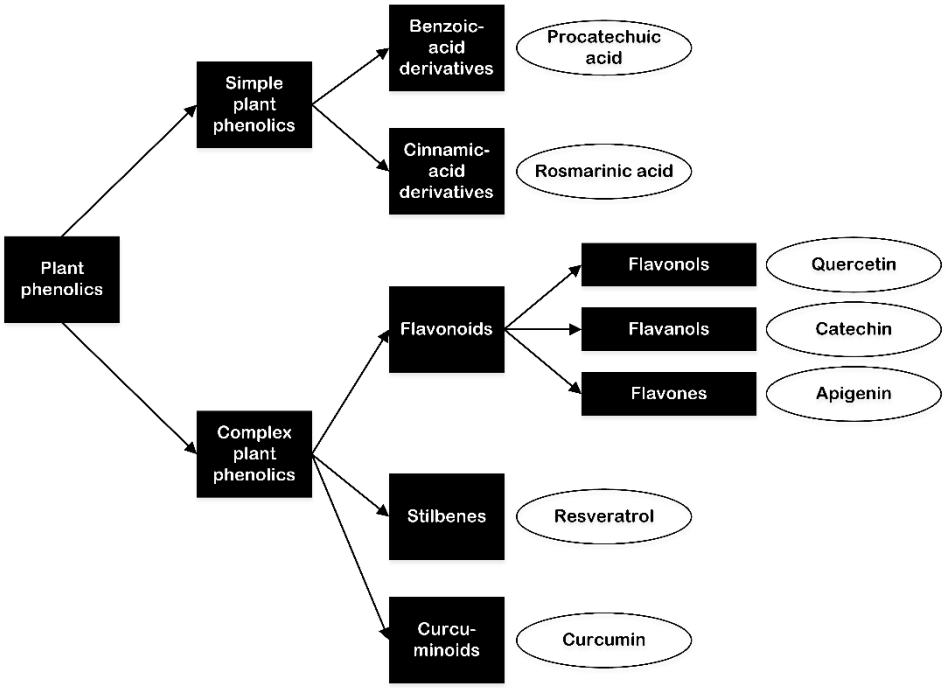

Figure 3. Classification of plant phenolics. Simple phenols contain only one aromatic ring, while complex phenols two or more aromatic rings. The main groups – flavonoids, phenolic acids, stilbenes and curcuminoids – and subgroups of flavonoids are selected as examples, depicting groups relevant to this study only. For each group or subgroup one example compound is presented. The figure is adapted from Anantharaju et al. (2016).

2.6 ACTIVITIES OF PLANT PHENOLICS POTENTIALLY BENEFICIAL TO HEALTH

Epidemiological studies and meta-analyses have indicated that there is an association between diets rich in fruits and vegetables and the reduction of chronic disease such as cancers, and neurodegenerative and cardiovascular diseases (Estruch et al. 2013, Hollman 2014, Zamora-Ros et al. 2010). According to Block and co-workers (1992), the protective effect of such diets against cancer was found in 128 of 156 studies. They indicated that persons with a low intake of fruits and vegetable had two-fold greater risk of cancer than persons with a high intake. Experience and knowledge of the use of plants for medicinal purposes can be found in traditional medicinal systems, such as traditional Chinese medicine and Indian Ayurveda (Liu et al 2015, Bhandaria et al. 2015). Several reviews and original studies discuss health-beneficial plants, their target diseases, mechanisms of action, evidence of their efficacy, and possible toxicity (see, for example, Bhandari et al. 2015, Liu et al. 2015). In Europe and other Western countries, the health-beneficial effects of plants have been discussed in several evidence-based monographs, for example in publications of the Committee on Herbal Medicinal Products (HMPC) by the European Medicines Agency (EMA), the European Pharmacopoeia, and World Health Organization (WHO) monographs. Examples of plants with different health benefits are presented in Table 2.

Plant phenolics have been adapted evolutionary to interfere with the cell signalling of pathogens in order to protect the plant. All plant-protecting compounds have a receptor-binding capacity that is not limited to pathogenic microbes alone but also interact with the molecular targets of mammals (Williams et al. 1989). Plant phenolics contain several reactive structures by means of which they are able to target human cellular receptors, enzymes, transcription factors, and DNA. They are able to change the binding ability and three-dimensional structure of proteins by non-selective covalent or non-covalent modifications (Wink 2015).

Natural products often lack the target specificity that could grant them a beneficial character when affecting cell signalling in human cells (Imming, Sinning and Meyer 2006). This unique pool of natural compounds cannot be replaced by synthetic compounds, and there is great potential to discover new modes and mechanisms of action to improve health or treat and prevent disease (Anantharaju et al. 2016).

Table 2. Examples of botanical drugs and therapeutics. [1]WHO 2017, [2]EMA 2006, [3]Mason 2009, [4]EFSA 2012, and [5]EFSA 2011.

Drug or therapy	Description	Product or plant (indication)
Drugs	Single molecule	Paclitaxel (cancers)[1]
Botanical drugs	Mixtures. Clinically validated and standardised.	Sweet fennel, fruit (gastro-intestinal complaints, cough)[2]
Dietary supplements	Plant components	Ginkgo biloba extract (memory)[3], green tea extract (antioxidant)[3], guarana (mental alertness)[3]
Functional foods	Food ingredient with beneficial effects on health.	Plant sterols and stanols (cholesterol)[4], xylitol (caries)[5]

2.7 ANTI-CANCER ACTIVITIES OF PLANT PHENOLICS

The anti-cancer activities of plant phenolics are claimed to be due to the combination of cytoprotective effects on healthy cells and cytotoxic effects on cancerous cells resulting from antioxidant, pro-oxidant, anti-inflammatory, and anti-clastogenic activities (Anantharaju et al. 2016, Ramos 2008). Alterations in oxidative balance in cancer cells activate

antioxidant functions accompanied by the up-regulation of pro-survival genes. This allows a constant oxidative-stress condition without triggering apoptosis (Acharya et al. 2010). A potential antioxidant-based anti-cancer mechanism of plant phenolics involves reducing oxidative cellular stress and supporting cells to commit self-destruct. The antioxidant properties of plant phenolics have been studied over a lengthy period (Halliwell 2001).

Recent research has focused on the mechanisms of plant phenolics involved in cell signalling and epigenetics (Ayissi, Ebrahimi and Schluesenner 2013; Millimouno et al. 2014). For example, curcumin and resveratrol have been shown to be capable of interfering in both mechanisms in human cells (Mai 2007). Plant phenolics have been demonstrated to target the cell signalling of cancer cells by inducing cell-cycle arrest, inhibiting oncogenic signalling in cell proliferation, apoptosis, and angiogenesis (Anantharaju et al. 2016). The molecular targets for inducing apoptosis include transcription factors such as p53, growth factors, tumour cell-survival factors, inflammatory cytokines, protein kinases, and proteins involved in angiogenesis (Millimouno et al. 2014). For example, curcumin – the main curcuminoid in rhizomes of *Curcuma longa* L., which has been used to treat inflammatory diseases in Ayurveda – is able to suppress the proliferation of various tumour cells by targeting several anti-tumorigenic molecular events. It up-regulates p53 and pro-apoptotic Bax (Li et al. 2015) and down-regulates anti-apoptotic and inflammatory NF-κB (Wang et al. 2014) and p53-inhibitor Mdm2 (Li et al. 2015). Curcumin is being intensively investigated as a potential drug candidate for cancer treatment.

Epigenetics, where gene expression can be affected by targeting chromatin modifications such as acetylation, methylation, and phosphorylation, is one of the most interesting of possible treatment scenarios. Some effects on cell signalling could be explained by interference of epigenetics. Regulators of histone acetylation, such as SAHA and FK228, are very similar to plant polyphenol compounds that

have shown to have beneficial effects via epigenetic modification (Rahman and Chung 2010).

2.7.1 Combination approach and synergism

Toxicity and other adverse effects of chemotherapeutic agents have highlighted the need for more tolerable, effective, and safe approaches to cancer treatment. During the last decade, the combination approach with its potentially additive or synergistic effects has become more popular for treating complex diseases such as cancer. For example, Yue et al. (2014) have demonstrated synergistic effects when combining aspirin and metformin to treat pancreatic cancers. The beneficial effects of synergism in combination approaches using two or more compounds are achieved using lower treatment concentrations, decreasing the toxicity of the active compounds. In several studies, plant phenolics have been shown to possess such effects. For example, curcumin has been tested for synergistic properties in combination with different drugs or drug candidates. Hossain et al. (2012) observed the apoptotic effects of low doses of curcumin and the anti-cancer drug paclitaxel in glioblastoma cells. The combination triggered apoptotic cell death by activating caspase 8, inducing the cleavage of Bid to tBid, increasing the Bax:Bcl-2 ratio, and triggering the release of cytochrome c, Smac, and AIF from mitochondria. The combined treatment decreased the viability of cancer cells more than either drug alone. Chen et al. (2014) demonstrated the synergism of curcumin and the natural terpene, borneol, in melanoma cells – the combination activated apoptosis by upregulating p53, Brca1, c-Jun N-terminal kinase (JNK), and serine-threonine protein kinase (ATM).

The combination approach has been the basis of traditional medicinal systems for centuries. Recently further evidence has been produced to support the involvement of synergism when using herbal medicinal products (Zhou et al. 2016). Due to their complex nature, the mechanisms of synergy are more challenging to define than those between two or three active compounds. Synergism between two plant extracts has also

been found and studied. For example, the combination of peppermint (*Mentha piperita* L.) and sage (*Salvia officinalis* L.) plant extracts decreased the viability of colon-cancer cells *in vitro* by means of synergistic effects (Yi and Wetzstein 2011).

2.7.2 Plant phenolics and p53

The transcription factor and tumour suppressor p53 is active in numerous cellular functions related to cellular stress, cell proliferation, and death. This broad involvement in cell survival and death makes it an interesting target for drug treatments. Numerous studies have demonstrated that plant phenolics and extracts are capable of activating p53 or p53-mediated pathways (Gonzales-Vallinas et al. 2013). Interestingly, the activation of apoptotic or anti-proliferative pathways is not dependent on p53 status as wild-type, mutant, or deficient. Activation of impaired p53 has been demonstrated for several plant phenolics, including curcumin, apigenin, and quercetin (see review by Etienne-Selloum et al. 2013).

Plant phenolics activate or interfere with p53 by means of various mechanisms. Molecular dynamics-simulation studies have demonstrated the ability of the plant phenolics taxifolin and quercetin to disrupt interactions between Mdm2 and p53 (Verma, Singh Mishra 2013) which prevents the degradation of p53. Another approach is to target post-translational modifications of p53. A combination of the two plant phenolics luteolin and (-)-epigallocatechin-3-gallate activated apoptosis and stabilised and increased the ATM-dependent phosphorylation of p53 following the translocation of p53 to the nucleus (Ruhul Amin et al. 2010). Apigenin has been demonstrated to induce both wild-type and mutant p53. Torkin et al. (2005) demonstrated that apigenin can induce the transcription activity of p53 followed by the expression of p21 and Bax, which activate apoptotic cell death in colon-cancer cells. King and co-workers (2012) showed that apigenin is able to induce apoptosis by means of mutated p53 by the translocation of p53 to the nucleus, DNA

binding, and the expression of p21 and Puma in two pancreatic cancer-cell lines.

2.7.3 Anti-cancer activities of *Juniperus communis* L.

Juniper (*Juniperus communis* L., Cupressaceae) is an evergreen conifer common in temperate regions of the northern, and in some parts of southern, hemisphere (Adams 2011). Juniper cones – commonly referred to as berries – are used as a game spice, or to flavour alcoholic beverages such as gin, and in cosmetic products. Uses for juniper berries for various ailments are reported in traditional medicine around the world (Table 3). Its anti-cancer uses are not reported simply due to a lack of use, or a lack of reporting due to the difficulties in diagnosing cancer in the past.

A Swedish study by Tunon, Olavsdotter and Bohlin (1995) evaluated the anti-inflammatory activities of aqueous extracts from traditional medicinal herbs, including juniper berries (*Juniperus communis* L.). Juniper berry extract showed moderate inhibition of prostaglandins – locally synthesised lipid compounds regulating inflammation – and platelet-activating factor exocytosis. Quercetin, the main phenolic of juniper, has been shown to sensitise cancer cells to apoptosis by activating AMPK via phosphorylation by tumour suppressor liver kinase B1 (LKB1), and further modulating the mammalian target of rapamycin (mTOR) pathway. That pathway, together with the activation of p53, promotes LKB1-dependent tumour suppression (Hardie 2011, Shackelford and Shaw 2009). There has been concern about nephrotoxicity of juniper berries in humans, which is claimed to be associated with volatile oil components. However, Schilcher and Leuschner (1997) did not detect any nephrotoxicity in rats after 28-days of oral administration of volatile oil of juniper, or terpinen-4-ol. Hepatotoxicity has not been found for a methanol extract of juniper berries in human HepG2/C3A cells and rat MH1C1 cells (Liu et al. 2011).

Table 3. Examples of traditional uses of juniper berries (*Juniperus communis* L.)

REGION	AILMENTS	REFERENCE
India	oedema, dropsy, sciatica, lumbago, rheumatism, swollen joints, diabetes, arthritis, weak digestion, dysmenorrhea and weakened immune system (Ayurveda)	Moerman (2009)
North America	urinary tract infections, kidney pain and disorders, respiratory ailments (coughs, sore throats, lung infections), tuberculosis, cold and fever, diarrhoea, stomach ulcers, and pain	Khalsa and Tierra (2008)
Sweden	wounds, swellings, pain, fevers, rheumatism, bites, and headache	Tunon, Olavsdotter and Bohlin (1995)
Turkey	cough, haemorrhoids, and pain	Fujita et al. (1995)

2.8 PLANT PHENOLICS IN DRUG DISCOVERY

Plant-derived natural compounds, including plant phenolics, may provide affordable drug candidates with the ability to target multiple cell-signalling pathways. The importance of natural remedies in drug development is indisputable: 51% of all new approved drugs from 1981 to 2014 were natural and botanical products, their derivatives, or synthetic drugs developed with the natural product as a basis (Newman and Cragg 2016). The reductionistic one-molecule-for-one-target approach to screen single molecules purified from plants by means of high-throughput bioassays is one of the most economical and time-saving approaches to search for potential drug candidates (Harvey, Edrada-Ebel and Quinn 2015). The limitation is that it ignores the complexity of biological functions, and any additive or synergistic effects of drug candidates cannot be detected. A step closer to a holistic view in drug discovery has been the screening of whole extracts or fragments of them.

Systems biology utilising, for example, metabolomics, proteomics, and metagenomics, serves as a tool for a holistic approach to search for complex multi-drug candidates. Different approaches are discussed in several reviews, for example, by Atanasov et al. (2015). The first hints of potentially active plant extracts can be found in dietary studies, the use of dietary supplements, and ethnopharmacological knowledge (Kinghorn et al. 2011).

2.8.1 Holistic and ethnopharmacological approaches

Our ancestors explored natural sources to search for cures for different ailments and diseases, and the difference between food and medicine was not as clear as it is today (Petrovska 2012). Today, the ethnobotanical approach to drug development is under challenge due to a loss of knowledge as a result of globalisation and modernisation (Vandebroak and Balick 2012). The mechanisms of action of traditional herbal medicinal products are often not understood; however, that does not mean there is no activity. Using ethnopharmacological information to search for possible drug candidates can be seen as a holistic approach in which 'clinical trials' are conducted before determining the molecular activity of the drug candidates. Finding traditional medicines for conditions such as wounds, fever, or snake bites is straightforward due to well-defined descriptions of use. For more complicated diseases, such as cancer, limited capabilities to diagnose the disease makes the identification of traditionally used herbal medicines more challenging, or even impossible.

2.8.2 Case study: curcumin

The plant phenolic curcumin, or turmeric extract, provides an interesting example of the drug discovery and development process. Several *in vitro* tests, animal tests, and clinical trials have revealed its efficacy against cancer, other ailments, and its supportive role in combination therapies. For example, curcumin has been shown to inhibit the cardiotoxicity of

doxorubicin by inducing autophagy and reducing apoptosis through the inhibition of JNK phosphorylation (Katamura et al. 2014). Recently published criticism (Baker 2017, Nelson et al. 2017) on the health benefits and suitability of curcumin as a drug has foregrounded the discussion of the methods and approaches of studying natural compounds and extracts. Heger (2017), on behalf of his co-workers, has argued in support of the observed effects of curcumin, and has criticised the one molecule, one target approach as unsuitable for natural compounds. He has also raised the issue of the unique character of the binding behaviour of curcumin as more modulatory than definitive. He has stated that the quantity and quality of evidence is sufficient to continue research with curcumin, but that more attention should be paid to methods and approaches when studying natural compounds. He has claimed that curcumin should be studied as a component of turmeric extract rather than as a single compound. These notices and claims could be applied to other plant phenolics as well.

2.9 DIETARY INTAKE OF PLANT PHENOLICS

Dietary intake of plant phenolics and especially flavonoids has been monitored in various regions of the world. The consumption of plant phenolics as they occur in herbs and vegetables is often seasonal, and intake is highest in summer when fresh vegetables and herbs are available. The main sources of the different phenols vary between countries. A daily mean intake of polyphenols 1756.5 ± 695.8 mg was measured in Krakow, Poland (Grosso et al. 2014). In total, 347 polyphenols from 19 different sub-classes were detected, though the main groups were flavonoids (897 mg/d) and phenolic acids (800 mg/d). In comparison, in France, the daily intake of polyphenols was estimated to be 1193 ± 510 mg (Perez-Jimenez et al. 2011).

Justesen and Knuthsen (2001) calculated the Danish intake of flavonoids from commonly used herbs in traditional Danish dishes. They found that

parsley is the richest herbal source of flavonoids in Danish dishes. Total intake of parsley flavonoids such as the flavones, apigenin and luteolin, and the flavonol, quercetin, varied from 5 to 47 mg per serving. The absorption and bioavailability of flavonoids was not determined. In more temperate Spain, the main sources and intake of flavonoids were apples, red wine, oranges, and other fruits, with a daily intake of 313 mg determined by Zamora-Ros and co-workers (2010). The most abundant flavonoids in the Spanish diet were proanthocyanidins (60%), flavanones (17%), and flavan-3-ols (10%). In the Australian diet, the dominant flavonoid sources were black and green tea for flavonols and flavan-3-ols, and citrus fruits for flavanones (Somerset and Johannot 2008).

2.10 BIOAVAILABILITY OF PLANT PHENOLICS

The bioavailability of dietary plant phenolics is a factor critical for their health-benefits. Although their bioavailability is relatively poor due to inadequate absorption, instability, and modifications by gut microbiota, their biological activity has been detected even at low concentrations (Abourashed 2013, Nifli et al. 2005). The maximum plasma concentrations are detected stepwise, with the first concentration peaks observed 1-2 hours after intake, and later when absorption occurs after modifications by colonic microflora (Scalbert and Williamson 2000). Aglycone forms are absorbed in the intestine, but in dietary intake, most of phenols occur as esters, glycosides or polymers, and they cannot be absorbed as such (D'Archivio et al. 2007). Bioavailability of plant phenolics is inevitable (Scalbert and Williamson 2000), but due to several metabolic modifications such as methylation, sulfation and glucuronidation, during absorption, and further by liver enzymes, the identification of metabolites and determination of bioavailability is made challenging (Day and Williamson 2001). Numerous studies have demonstrated the bioavailability of such plant-derived molecules, and recently the results have been summarised in several reviews, for example, by Abourashed (2013).

2.11 SAFETY OF PLANT PHENOLICS

Plant phenolics are generally considered safe when consumed in fruits and vegetables; however, concentrated amounts of plant phenolics in extracts, or pure phenols ingested as food supplements or herbal medicines may cause systemic toxicity in humans (Anantharaju et al. 2016). The poor bioavailability of plant phenolics provides a natural mechanism to avoid toxicity when ingested in high concentrations, however, for medicinal use the bioavailability of active compounds needs to be confirmed. Mechanisms to enhance bioavailability of drugs have been introduced, however, the toxicity of higher concentrations of plant phenolics limits the use of such systems (Tabrez et al. 2013). For example, higher concentrations may contribute to the development and progress of cancer instead of to the observed anti-cancer activities at low concentrations (Fresco et al. 2013). This observation supports the method of intake of plant phenolics by dietary means or by means of extracts with lower plant phenolic concentrations.

Parallel use of drugs and herbal products may cause adverse interactions between them. The most common pharmacokinetic alterations to administered drugs caused by plant phenolics are mediated via CYP enzymes (Wanwimolruk and Prachayasittikul 2014). For example, on the safety of resveratrol as a novel food, the opinion of European Food Safety Authority (EFSA) was that it is safe, but may inhibit CYP enzymes and interact with drugs metabolised by CYP2C9 (EFSA 2016).

There is not centralised authorisation procedure in the EU for the use of plant products such as plant extracts as food supplements. However, the general safety requirements of EU food law, set out in EU Regulation (EC) No. 178/2002, apply to plant products. When the product is used for medicinal purposes as a traditional herbal medicine, the EMA assesses the safety of the product in terms of Herbal Directive (EC) No. 24/2004. For substances and mixtures not covered by specific legislation, the identification of hazards and classification is required in terms of EU CLP

Regulation (EC) No 1272/2008. For example, curcumin (synthetic) has been self-classified by a supplier as an eye and skin irritant, and resveratrol as a skin and respiratory irritant, harmful to aquatic life, and suspected of causing genetic defects and serious eye damage. Hazard and risk-assessment methods of chemicals are mainly designed for single substances, and therefore the assessments of the authorities often lack information on the synergistic and additional effects of mixtures such as plant extracts and other herbal products.

3 AIMS OF THE STUDY

The general aim of this study is to investigate plant extracts and phenols for their effects on cell death and cell signalling associated with cancer. To achieve this aim, molecular- and cell-biology analyses describing cell death and apoptosis were performed on cancerous and non-cancerous cell models.

The specific aims of this study are:

- To assess the predictability of the cytotoxic effects of plant extracts based on their known antioxidant activities (I-III, unpublished)
- To compare the sensitivity of cancerous and non-cancerous cell models to treatment with plant phenolics and extracts (I, III)
- To evaluate the effects of plant phenolics and extracts on p53, and other proteins involved in the signalling of cell death (I, II, IV)
- To further investigate a selected plant extract for its cytotoxic and cell death-inducing effects, potential synergism, and the phenolic content (IV)

4 EXPERIMENTAL

A detailed description of materials and methods is presented in the original publications I-IV.

4.1 CELL CULTURES

4.1.1 SH-SY5Y – Human neuroblastoma cells

The human SH-SY5Y cells are tumorigenic epithelial neuroblastoma cells originated from a metastatic bone marrow tumour in a 4-year-old female (Biedler et al. 1978). SH-SY5Y cells are commonly used as a dopaminergic cell model (Xie, Hu and Li 2010), and possess sensitivity for use in toxicological treatments (Puttonen et al. 2008). In this study, SH-SY5Y cells were used as a sensitive tumorigenic cell model. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) : Ham's nutrient mixture F-12 (1:1) containing 15 mM HEPES buffer and L-glutamine, supplemented with 15% (v/v) heat-inactivated foetal bovine serum, penicillin (170 U/ml), streptomycin (170 µg/ml), and 1% non-essential amino acids (Gibco BRL). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2in air. The culture medium was replaced every 3-4 days, and the cells were sub-cultivated once a week.

4.1.2 A375 – Human melanoma cells

The human A375 cells are tumorigenic epithelial melanoma cells originated from a malignant skin tumour in a 54-year-old female (Giard et al. 1973). The cells were obtained from ATCC (CRL-1619). In this study, A375 cells were used as a tumorigenic cell model. The cells were cultured in DMEM containing GlutaMAX I, 4,500 mg/l glucose and 110 mg/l sodium pyruvate, and supplemented with 10% (v/v) heat-inactivated foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco BRL). The cells were maintained at 37 °C in a humidified atmosphere

containing 5% CO_2 in air. The culture medium was replaced every 2-3 days, and the cells were sub-cultivated twice a week.

4.1.3 CV1-P – African green monkey fibroblast cells

The African green monkey CV1-P cells are non-tumorigenic fibroblast cells originated from a kidney of an adult male monkey, and were obtained from the University of Eastern Finland. CV1-P cells are commonly used for transfections (Gjoerup, Zaveri and Roberts 2001) and as a non-neuronal cell model (Manakova et al. 2003). In this study, the CV1-P cells were used as a non-tumorigenic cell model. The cells were cultured in DMEM containing L-glutamine, 1,000 mg/l of D-glucose and sodium pyruvate, and supplemented with 10% (v/v) heat-inactivated foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco BRL). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. The culture medium was replaced every 2-3 days, and the cells were sub-cultivated twice a week.

4.1.4 Treatments

All cell lines were cultured in 96-well plates for cell-viability assays, in 6-well plates or 60 mm culture dishes for Western blot analyses, DNA fragmentation, and caspase 3 activity tests. The SH-SY5Y cells were cultured and treated on 10 cm plates for cDNA-representational difference analysis (RDA) assay. The cells were exposed to compounds and plant extracts 18-24 hours after plating. AraC, an apoptosis-inducing control compound, was dissolved in MilliQ H_2O, curcumin and resveratrol in DMSO:MilliQ H_2O (1:1), and plant extracts in a cell-culture medium, MilliQ H_2O, DMSO, or methanol depending on their solubility. The final concentration of DMSO varied between 0.1-1.5% (v/v) and the methanol concentration was 1% (v/v). The control cells were exposed to the same amount of DMSO or methanol as the treated cells. Plant phenolic treatment concentrations varied from 5 to 400 µM, and plant extract concentrations from 10 to 2000 µg/ml. Cell viability assays were

performed immediately after treatments. Other analyses were either performed immediately or the cells were stored at -80 °C.

4.2 PLANT MATERIAL

Resveratrol (trans-resveratrol, R5010) and quercetin (Q4951) were obtained from Sigma-Aldrich. Curcumin (> 95% in turmeric-extract powder T-14, Lihel code 3508) was obtained from Lihel, Finland. Control compound cytarabine (AraC) (C1768, cytosine ß-D-arabinofuranoside free base) was obtained from Sigma-Aldrich. Aqueous plant extracts from basil leaves (*Ocimum basilicum* L., laurel leaves (*Laurus nobilis* L.), juniper berries (J*uniperus communis* L.), and parsley leaves (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill) were prepared by Hinneburg, Dorman and Hiltunen (2006) with the hot-water extraction method followed by the removal of volatile oils by hydro-distillation. Extracts were freeze-dried and stored at 4 °C. Dried plant material was obtained from Oy Gustav Paulig Ab, Finland. Methanol extract of parsley leaves (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill) was prepared by maceration with MeOH:H_2O : HOAc (80 : 20 : 1, v/v/v) followed by defatting using petrol and lyophilisation. Air-dried leaves of parsley were obtained from Pimenta Oy, Finland. The extract was stored at 4 °C. Lemon balm leaf (*Melissa officinalis* L.) extract was prepared by Dastmalchi et al. (2008), with aqueous ethanol (450 ml/l) using medium-pressure liquid-solid extraction. The plant material (Specimen No. GeLM175) was obtained from the Institute of Medicinal Plants, Tehran, Iran. Siberian pine seed (*Pinus sibirica* Du Tour) extract was prepared by defatting with hexane and macerated with acetone:water (95 : %, v/v) at 45 °C. The Siberian pine seeds were obtained from Vigado, Moscow, Russia. The extract was freeze-dried and stored at 4 °C.

4.3 CELL VIABILITY

4.3.1 MTT assay

An assay of tetrazolium dye (MTT) was used to examine the metabolic activity of the cells. The MTT assay is based on the reduction of the yellowish MTT to dark-blue formazan by viable and metabolically active cells. The reduction has been demonstrated to occur as a result of the activity of mitochondrial dehydrogenases (Mosmann 1983), but also as a result of enzymatic activity outside mitochondria (Liu et al. 1997, Takahashi et al. 2002). The assay was performed by adding an MTT powder (Sigma) dissolved in Hank's balanced salt solution or PBS to the treated and control cells to attain a final concentration of 0.5 mg/ml. SH-SY5Y and A375 cells were incubated with MTT for 2.5 hours, and CV1-P cells for 2.5-4 hours in culturing conditions. Formazan crystals were dissolved in DMSO and the absorbance was measured at 560 nm, with the background subtracted at 655 nm, using a microplate reader (Bio Rad, model 550, Japan). The percentage of metabolic activities were calculated from the values of two to four independent experiments using the equation: % metabolic activity = [(absorbance of treated cells) / (absorbance of untreated cells)] x 100.

4.3.2 LDH assay

The membrane integrity of cells was examined by a lactate dehydrogenase (LDH) assay using a commercial kit, CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega). The fluorometric assay is based on the conversion of resazurin into fluorescent resorufin by the enzymatic activity of LDH leaked from the cells through damaged membranes. Measurements of the activity of LDH indicate the number of non-viable cells, and have been used to detect necrotic cell death (Chan, Moriwaki and De Rosa 2013). The assay was performed according to the manufacturer's instructions. In brief, equal amounts of medium from treated and control cells were mixed with CytoTox-ONE reagent. After

incubation, the enzymatic reaction was stopped by adding a CytoTox Stop Solution. The fluorescence was determined at an excitation wavelength of 560 nm and an emission wavelength of 590 nm, using a fluorescence microplate reader (Varioskan, Thermo Fisher Scientific). The percentage of membrane integrity was calculated from the values of three to four independent experiments using the equation: % membrane integrity = [(experimental-blank) / (control-blank)] x 100, where the blank refers to the background, measured for the medium alone.

4.4 PROTEIN EXTRACTION AND QUANTIFICATION

4.4.1 Total protein extraction

Proteins were extracted from treated and control cells for Western blot analysis by incubating cells with a lysis buffer containing 20 mM HEPES, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.1% Triton X-100, and supplemented with a cocktail of protease inhibitors (Complete Mini, Roche). After incubation, the extraction process was supported with sonication and the total protein was separated by centrifugation at 16,000g for 15 minutes for proteins from SH-SY5Y cells, and at 13,000g for 30 minutes for proteins from CV1-P cells. For the caspase 3 activity assay, proteins from A375 cells were extracted using a lysis buffer containing 10 mM Tris-HCl, 10 mM $Na_2H_2PO_4$, 10 mM Na_2HPO_4, 1% Triton X-100, with pH 7.5. Proteins were separated by centrifugation at 20,000g for 15 minutes. All total-protein extraction steps were performed on ice or at 4 °C.

4.4.2 Cytoplasmic and nuclear protein extraction

Nuclear and cytoplasmic proteins were extracted by using a commercial kit, ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas). The extraction was performed according to the manufacturer's instructions. In brief, cytoplasmic proteins were extracted by incubating cells with a cell-lysis buffer supplemented with protease

inhibitors. Proteins were separated from nuclei by centrifugation at 1,000g for 10 minutes. Nuclear proteins were extracted from washed nuclei with a nuclei lysis reagent and separated by centrifugation at 16,000g for 15 minutes. All extraction steps were performed on ice or at 4 °C. Protein samples were stored at -80 °C.

4.4.3 BCA protein assay

Protein concentrations were examined by using a colorimetric bicinchoninic (BCA) protein-assay kit (Pierce, Thermo Scientific). The method is based on the capability of proteins to reduce a copper-containing reagent which reacts with BCA, forming a purple complex with an absorbance maximum at 550 nm. Protein samples for the assay were quantified immediately after extraction or stored at -80 °C. The assay was performed according to the manufacturer's instructions. In brief, extracted proteins and standard bovine serum albumin samples were diluted with the mixture of assay buffers in duplicate in 96-well plates. The absorbance of samples was read using a microplate reader (Bio Rad, model 550) at a wavelength of 550 nm. Protein concentrations were determined by comparing the values with those of the standardised protein samples. For Western blot analysis, the samples were diluted to the same concentration, and for the caspase 3 activity assay the samples were used as such. Protein samples were used immediately for the caspase 3 assay and Western blot analysis, or stored at -80 °C for Western blot analysis.

4.5 MECHANISMS OF CELL DEATH

4.5.1 Caspase 3-like activity

Caspases are the key regulators of apoptotic cell death. Their involvement in the observed loss of viability of A375 cells was quantified by the cleavage of a fluorogenic substrate, Ac-DEVD-AMC, specific to caspases 3 and 7. The assay was performed as described by Manakova et al. (2005)

and Ossola et al. (2011). In brief, enzymatic reaction was induced by mixing equal amounts of protein sample and assay buffer (20 mM HEPES, 10% glycerol, pH 7.5) containing the Ac-DEVD-AMC substrate and dithiotreitol (DTT). After incubation for 2 hours at 37 °C, the cleavage of the caspase-specific substrate was measured with excitation at 370 nm and emission at 445 nm using a fluorescence microplate reader (Varioskan, Thermo Fisher Scientific). The results were calculated as the release of AMC in pmol/min/mg of protein using a calibration curve for AMC.

4.5.2 DNA fragmentation

Another apoptosis-related biochemical feature of cell death is the fragmentation of DNA. Fragmentation was determined by agarose gel electrophoresis as previously described by Matassov et al. (2004), with minor modifications. In brief, cells were collected and lysed using a lysis buffer (0.2% Triton X-100; 10 mM Tris-HCl, pH 7.5, and 10 mM EDTA in water). Samples were treated with RNAse enzyme and DNA was extracted using a phenol/chloroform method. The DNA was dissolved in a TE buffer (10 mM Tris-HCl, pH 8.0, and 10 mM EDTA in water) and mixed with 6x DNA loading dye for electrophoresis. DNA fragments were separated in 2% agarose gel in TAE buffer for 3 hours using a voltage of 100V. The image was photographed with a charged-coupled device camera (UltraLum) under UV light.

4.5.3 Western blot analysis of p53, Bcl-2 and p65

Protein samples from whole cells, cytoplasm or nuclei were separated using the sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The method is capable of high-resolution separation of polypeptides by their molecular mass. Conditions to break disulfide bonds of proteins for separation in the gel were made with β-mercaptoethanol or dithiotreitol (DTT). After the separation through the gel of 10-12%, proteins were transferred onto a nitrocellulose membrane.

The nonspecific binding of antibodies on the membrane was blocked by incubating membranes in TBS supplemented with 0.05% Tween 20 and 5% non-fat milk powder for 2 hours at room temperature.

Proteins p53, Bcl-2 and p65, and loading controls ß-actin and TBP were detected using mouse monoclonal primary antibodies to p53 (1:1,000, DO-7, Novocastra), Bcl-2 (1:2,000, SC-7382, Santa Cruz Biotechnology), NF-κB p65 (1:500, sc-8008, Santa Cruz Biotechnology), ß-actin (1:3,000, A1973, Sigma), and TBP (1:1,000, ab51841, Abcam). Antibodies were incubated with the proteins on the nitrocellulose membrane overnight at 4 °C in TBS supplemented with 0.5% non-fat milk powder for samples from A375 and SH-SY5Y cells, and with 5% non-fat milk powder for samples from CV1-P cells. The membranes were washed with TBS with 0.5% Tween 20, and exposed to a secondary antibody, an anti-mouse IgG-HRP (1:2,000, HAF007, RandD Systems) for 1 hour at room temperature. The proteins were visualised using a chemiluminescent reagent (SuperSignal West Pico, Thermo Scientific) by GeneSnap with GeneGnome (Syngene). The results were analysed using the GeneTools (Syngene) programme.

4.6 GENE EXPRESSION

4.6.1 Total RNA extraction and cDNA synthesis

Total RNA was isolated from juniper berry extract-treated and -untreated control cell samples by the TRIzol method (Gibco BRL), and the samples were stored at -80 °C. The concentration, purity, and integrity of total RNA were verified in the 1.5% agarose gel by spectrophotometry. Double-stranded cDNA was synthesised from the total RNA by SuperScript™ Double-Stranded cDNA Synthesis kit (Invitrogen) according to the manufacturer's instructions.

4.6.2 cDNA Representational difference analysis

Representational difference analysis (RDA) was performed as previously described by Hubank et al. (2004) with some modifications. In brief, ds-cDNA was digested with DpnII-enzyme (R0543S, BioLabs) and ligated to desalted R-Bgl-12/24 linkers (TAG Copenhagen) to enable the polymerase chain reaction (PCR) amplification with R-Bgl-24 primer (TAG Copenhagen). The PCR products were cut by DpnII-enzyme to remove R-linkers and to produce a driver sample which represents the gene expression of untreated control cells. A tester sample representing the gene expression of juniper berry extract-treated cells was prepared from cut PCR products by purifying R-linkers from the sample by gel electrophoresis and ligating cDNA to J-Bgl-12/24 linkers (TAG Copenhagen). The first difference products (DP1) were generated by hybridising a mixture of tester and driver at a ratio of 1:100 followed by an exponential PCR amplification of tester:tester hybrids. Driver:tester and driver:driver hybrids were eliminated. To remove false annealing products, second difference products (DP2) were produced. The DP1 were cut, ligated to N-Bgl-12/24 linkers (TAG Copenhagen) and hybridised with the driver at a ratio of 1:2,000. After amplification by PCR, the DP2 were cut with DpnII-enzyme, separated, and purified by gel electrophoresis, and ligated with BamHI-digested pGEM plasmid vector (Fermentas). The DP2 were cloned by transferring vectors into *Escherichia coli* DH10B (TOP10, Invitrogen) by electroporation. Vectors were purified and the analysed by cycle sequencing using M13 forward primers (Applied Biosystems). Sequences of differentially expressed genes were analysed in BLASTN 2.2.27+ using human G+T databases. Names and synonyms of genes were clarified using the database of the Hugo Gene Nomenclature Committee, and predicted proteins were identified using UniProtKB and Swiss-Prot databases. Possible interactions of predicted proteins were assessed using the STRING 9.1 database accompanied by a literature search.

4.6.3 Statistical analysis

The results are shown as arithmetic means ± SEM or ± SD. Statistical analyses were performed either by one-way ANOVA, followed by Dunnett's multiple comparison test or Tukey's test using the software GraphPad Prism 4.0 or 5.0 (GraphPad Software Inc) or by a one-tailed Student's test. Results with $p < 0.05$ were deemed significant.

5 RESULTS

5.1 GENERAL

Cytotoxicity and biochemical methods were applied and established in order to investigate plant phenolics and extracts for their cytotoxic and molecular effects on cell death in cancerous and non-cancerous cells. Cell viability and toxicity were determined using three different methods: microscope, MTT, and LDH assays. By means of microscopic examination, changes in the shape and size of cells were detected. The MTT assay measured the metabolic activity of cells. Decreased activity indicated reduced viability of cells but did not describe the reasons or the type of cell death. Therefore, the permeabilisation of the cell membrane was analysed by LDH assay. This measures the leakage of intracellular molecules through the broken cellular membrane. In apoptosis or other regulated cell-death types, the leakage of cellular contents is prevented. When combining these two assays, it is possible to explain whether reduced cell viability indicates regulated or accidental necrotic cell death.

To explain changes in cell viability, cell signalling related to p53 was examined by determining its quantity in cells using Western blotting assay for total protein samples as well as for the nucleus and cytosol. P53 is a versatile regulator of the cell cycle and death and is triggered by several types of cellular stress. Activation of p53 leads to cell-cycle arrest or cell death. For its general role as a response to cellular stress, p53 was selected as a molecular marker of cell response to treatment. Decisions to continue with further experiments with different plant-derived samples were made as a result of detected effects on cell viability and p53.

Although decreased metabolic activity, intact cell membranes and an increase of p53 suggests apoptosis or other regulated cell death, additional experiments are required to better understand cellular

responses to treatment. Selected plant phenolics and extracts were analysed for their effects on the fragmentation of DNA, caspase 3 activity, anti-apoptotic responses via the protein Bcl-2, and anti-inflammatory responses via p65. Differences in gene expression were studied by means of the cDNA RDA method, which reveals the differences between treated and non-treated cells. While other test methods were focused on the known targets of cell viability and cell death, the cDNA RDA method is able to detect novel changes at the mRNA level.

5.2 PLANT PHENOLICS

The results of studies of plant phenolics are summarised in Table 4.

Table 4. The effects of treatments with 1-50 µM of plant phenolics and 50-100 µM of the control compound cytarabine in cancerous SH-SY5Y and A375 cells and non-cancerous CV1-P cells. Metabolic activity was investigated by MTT test and the amount of p53 by Western blotting. Results with higher treatment concentrations are not presented in this table.

TEST MATERIAL	**PHENOLIC CLASS AND SUBCLASS**	**SH-SY5Y**		**A375**		**CV1-P**	
		MA	p53	MA	p53	MA	p53
Curcumin	Diarylheptanoid Curcuminoid	▲	▲	na	na	◄►	◄►
Resveratrol	Stilbene Stilbenoid	◄►	◄►	na	na	▼	◄►
Quercetin	Flavonoid Flavonol	na	na	▲	▲	na	na
Cytarabine		◄►	▲	▼	▲	◄►	▲

MA = metabolic activity (MTT test), p53 = amount of p53, ▲ = increase, ▼ = decrease, ◄► = no effect, or effects with higher treatment concentrations, na = not analysed.

5.2.1 Curcumin and resveratrol

Curcumin and resveratrol were tested in cancerous SH-SY5Y and non-cancerous CV1-P cells for their effects on cell viability and p53 (I). The treatment time was 12 hours and treatment concentrations varied from 5 to 100 µM. Curcumin significantly decreased the metabolic activity of cancerous SH-SY5Y cells and increased the total amount of p53 in them. Similar effects were not detected in non-cancerous CV1-P cells, except with four-fold treatment concentration. Resveratrol was shown to be slightly more toxic to non-cancerous CV1-P cells than cancerous SH-SY5Y cells. Metabolic activity decreased in both cell lines but the change was not statistically significant. Treatment with 100 µM significantly increased the amount of p53 in both cell lines and the change was larger in CV1-P cells.

5.2.2 Quercetin

Quercetin was tested in cancerous A375 cells for its cytotoxic, anti-inflammatory, and apoptotic effects (unpublished). The treatment time was 24 hours, and the treatment concentrations varied from 1 to 200 µM. Metabolic activity measured by MTT reduction decreased significantly, by 20-50%, after treatments with 25-200 µM concentrations. Membrane integrity decreased significantly only after treatment with 200 µM (Figure 4).

In Western blot analyses, the amounts of cytosolic p53 and Bcl-2, and of nuclear p53 and p65 were defined after 24 hours of treatment with different concentrations of quercetin (Figure 5). Quercetin significantly decreased the anti-apoptotic Bcl-2 in the cytosolic fraction for all treatment concentrations, increased the amount of p53 in both the cytosol and nucleus, and activated caspase 3 at concentrations 25 and 50 µM (Figure 6). The increase in the amount of p53 was not statistically significant due to variations of results. Quercetin did not have anti-inflammatory effects on p65.

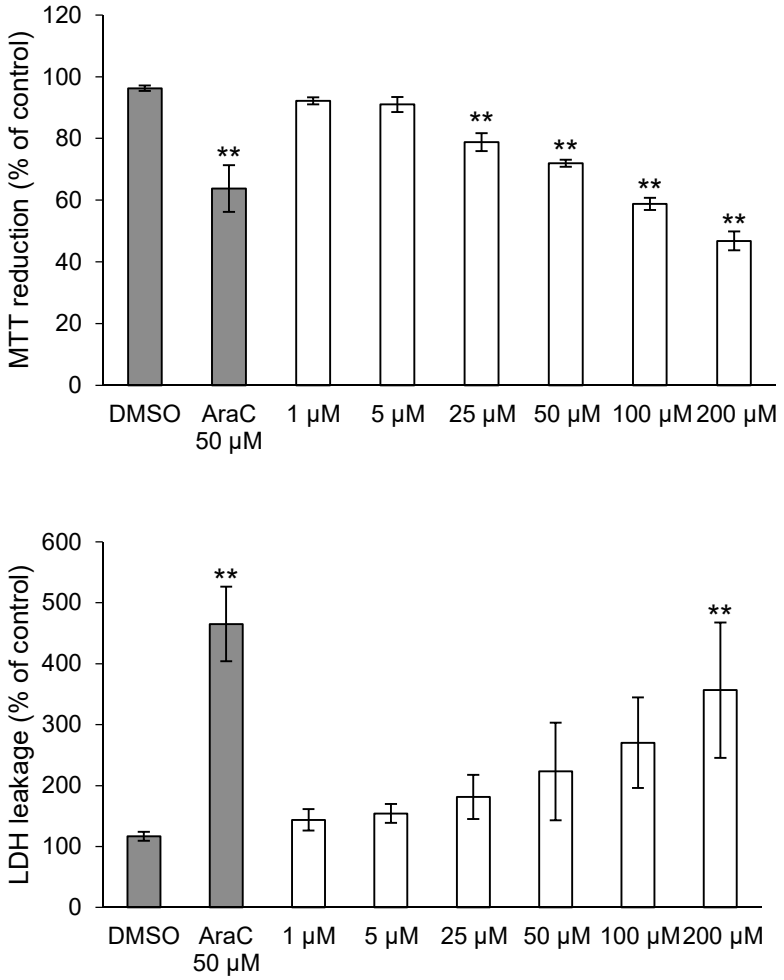

Figure 4. Metabolic activity (MTT) and membrane integrity (LDH) of A375 melanoma cells after 24 hours of treatment with quercetin. Data are presented as mean values ± SEM. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test: **$p < 0.01$.

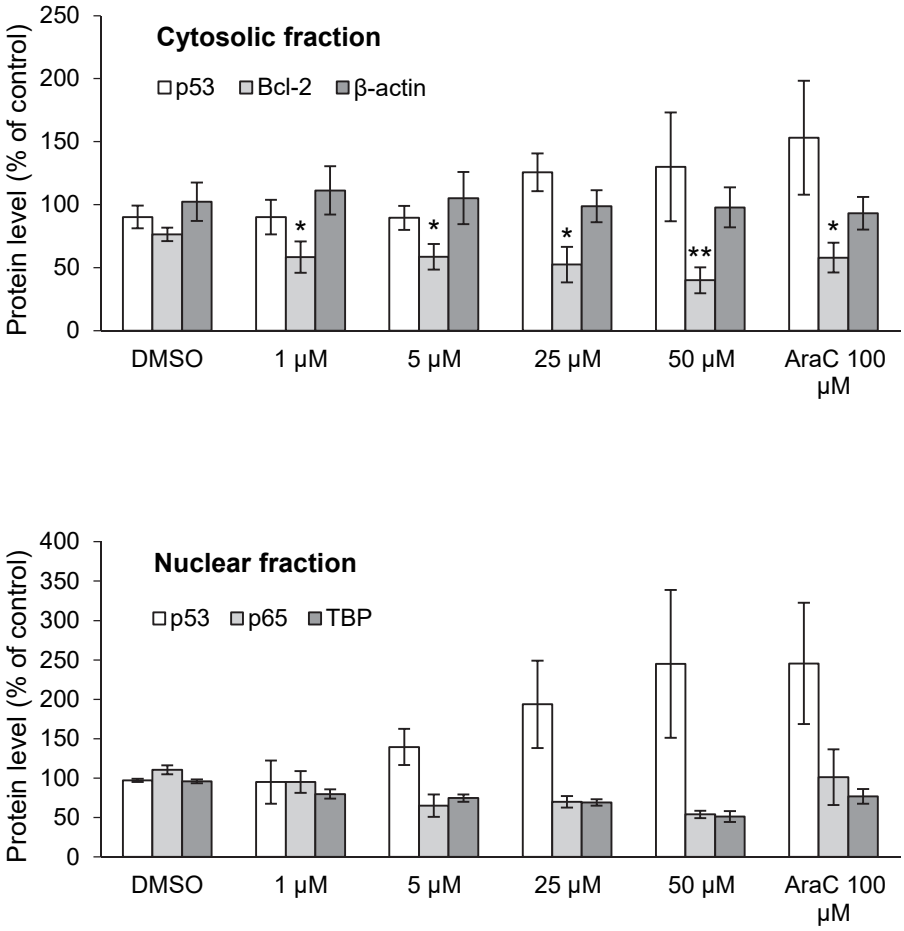

Figure 5. Amounts of cytosolic p53 and Bcl-2, and nuclear p53 and p65 in A375 melanoma cells after 24 hours of treatment with quercetin and AraC. Data are presented as mean values ± SEM. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test: *$p < 0.05$, **$p < 0.01$.

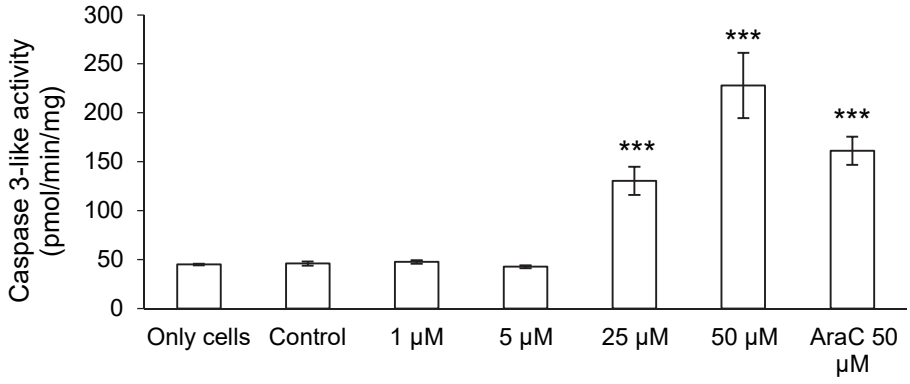

Figure 6. Caspase 3-like activity of A375 melanoma cells after 24 hours of treatment with quercetin and AraC. Data are presented as mean values ± SEM. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test: ***$p < 0.001$.

5.2.3 Cytarabine (AraC) – control compound

The chemotherapy agent cytarabine (AraC) was used as a positive control due to its known effects on cell viability, apoptosis, and p53 in SH-SY5Y and CV1-P cell lines in order to establish the cell models for the assays in this study. The effects on metabolic activity and p53 were tested with AraC concentrations ranging from 5 to 100 µM (I); in the other studies of this work, concentrations of 50 and 100 µM were applied as a positive control. Treatment times varied from 12 to 24 hours. The amount of p53 increased in all cell lines after AraC treatment, and the increase was almost two-fold in cancerous SH-SY5Y and A375 cells, compared to non-cancerous CV1-P cells (I, Figure 4). Treatments decreased both the metabolic activity (40%) and membrane integrity of A375 cells (Figure 4), but showed no effects on the metabolic activity of CV1-P and SH-SY5Y cells (I).

5.3 PLANT EXTRACTS

The results from the studies using extracts are summarised in Table 5.

Table 5. Summary of the effects of basil, juniper, laurel, parsley, and Siberian pine extracts, and information on the extracts, their phenolic content and antioxidant capacity, measured with free radical scavenging assay (DPPH).

		BA	**JU**	**LA**	**LB**	**PA**	**PA**		**SP**
Plant part		Leaves	Berries	Leaves	Leaves	Leaves	Leaves		Buds
Extraction type		H_2O	H_2O	H_2O	EtOH	H_2O	MeOH		Ac
Total phenols*		147 ± 1.60[1]	18.5 ± 0.62[1]	92.0 ± 2.45[1]	268.9 ± 21.3[2]	29.2 ± 0.44[1]	18.25 ± 1.21		266 ± 3.9
IC50 DPPH**		0.5	2.25	0.5	0.13	12	20		0.26
Cell line	N	■	■	■	■	■			■
	M						■		
	F							■	
Cell viab.	MTT	◄►	▼	▼	▼	◄►	▼	◄►	▼
	LDH	◄►	◄►	◄►	◄►	◄►	▲	◄►	▲
Necrosis (assumed)		yes	no	yes	(yes)	no	no	no	yes
p53		▲	▲	▲	◄►	◄►	na	na	◄►
DNA fragm.		na	▲	na	na	na	na	na	na
Casp. 3		na	na	na	na	na	◄►	◄►	na
Gene expr.		na	▲	na	na	na	na	na	na

BA = basil, JU = juniper, LA = laurel, PA = parsley, SP = Siberian pine. *Total phenolic content estimated as gallic acid equivalents (mg gallic acid / g extract dry weight), **Antioxidant activity estimated by the inhibition of free radical DPPH (IC50, mg/ml). [1]Hinneburg et al. (2006), [2]Dastmalchi et al. (2008). Cell lines: N = human SH-SY5Y neuroblastoma cells, M = human A375 melanoma cells, F = monkey CV1-P fibroblast cells. ▲ = increase, ▼ = decrease, ◄► = no effect, or effects with higher treatment concentrations, na = not analysed.

5.3.1 Predictability of cellular effects of antioxidant activity

The total phenolic content, antioxidant activity, and cellular effects of the plant extracts are summarised in Table 5. Direct predictability of cell-signalling effects was not detected, however, the exposure of cells with high concentrations of extracts to high antioxidant activity caused massive cell death with or without p53 activation, as determined by microscopic examination. The detected cytotoxicity was assumed to be necrotic for plant extracts from basil, laurel, lemon balm, and Siberian pine.

High total phenolic content predicted stronger antioxidant activity of an extract measured with the DPPH free radical scavenging method. The concentrations used to inhibit free radicals varied from 0.13 to 0.5 mg/ml for the basil, laurel, lemon balm, and Siberian pine extracts with the total phenolic content at 92.0-268.9 mg gallic acid/g extract. The low total phenolic content of parsley extracts indicated weak antioxidant activity. Juniper extract was the exception, with low total phenolic content and relatively high antioxidant activity.

5.3.2 Basil, laurel, lemon balm, and Siberian pine

Necrotic effects were observed only for Siberian pine extract at low treatment concentrations (II), whereas only high concentrations of laurel, basil, and juniper were necrotic (I). Laurel extract was toxic to cells at the highest treatment concentration (2,000 μg/ml) with visible shrinkage of cells (Figure 8) preventing testing of metabolic activity and p53. Laurel already decreased the metabolic activity at lower concentrations. Basil, laurel, lemon balm, and Siberian pine extracts significantly decreased the metabolic activity of cancerous SH-SY5Y cells (I, II, Figure 7). However, basil and lemon balm extracts showed this activity only at the highest treatment concentrations (800-2,000 μg/ml). The effect of basil extract was combined with a significant decrease in membrane integrity and an increase of p53 (I). The effects of Siberian pine extract were necrotic with

the decreased membrane integrity at all tested concentrations (200-1,000 µg/ml) without effects on p53 being detected (II).

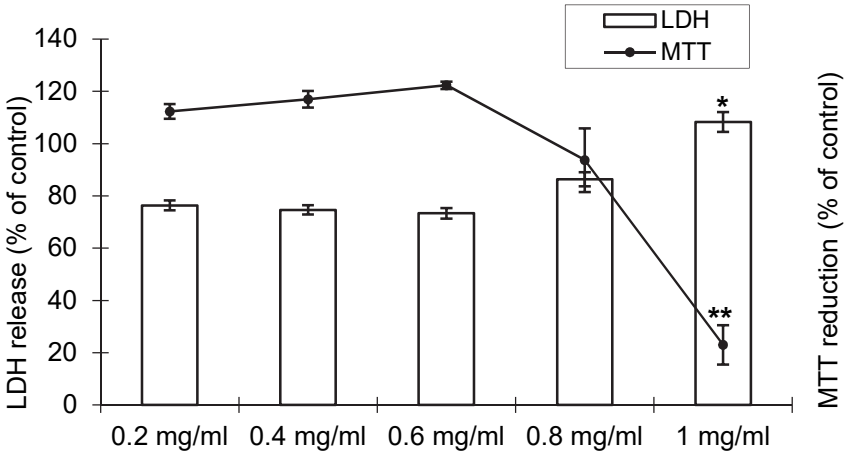

Figure 7. Effects of lemon balm extract on the metabolic activity and cell membrane integrity of SH-SY5Y cells. Results are from three independent experiments made in triplicate ± SEM (unpublished). Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test: *$p < 0.05$, **$p < 0.01$.

5.3.3 Parsley

Aqueous and methanol extracts of parsley were studied for their effects on cell viability, p53, and caspase 3 activity (I). Aqueous parsley extract had no effect on cell viability or the amount of total p53 in tumorigenic SH-SY5Y cells. However, the methanol extract decreased the metabolic activity and membrane integrity of cancerous A375 cells, though it did not have effects on non-tumorigenic CV1-P cells (III). Only the highest testing concentration of 2,000 µg/ml decreased the membrane integrity of non-cancerous CV1-P cells. The detected toxic effects of methanol extract were not related to caspase 3 -dependent apoptosis.

5.3.4 Juniper

5.3.4.1 Cell viability and p53

Aqueous extract of juniper berries was tested for cell viability in cancerous SH-SY5Y cells using the dose-response method. The metabolic activity of cells, measured by an MTT test, decreased with all treatment concentrations (0.001-2.0 mg/ml) but membrane integrity, measured by an LDH test, decreased only with higher treatment concentrations (I). Further studies have shown that the total amount of p53 in cells increased after treatments with 7.5-15 µg/ml (I) and that p53 was relocalised in the nucleus after 12 hours of treatment, although the results were not statistically significant (IV). The morphology of cells was assessed by microscopic examination (Figure 8). Compared to non-treated control cells, juniper-treated cells were smaller and shrunken (IV).

Figure 8. SH-SY5Y cells after treatment with juniper and laurel extracts. On left, non-treated control cells; in the middle, cells treated with 10 µg/ml of juniper berry extract; and on right, cells treated with 500 µg/ml of laurel extract.

5.3.4.2 DNA fragmentation

DNA fragmentation is a biochemical phenomenon of apoptosis. Juniper berry extract resulted in cleavage of DNA in SH-SY5Y cells after 24 to 72 hours of treatment (IV). Similar effects in non-treated cells were not detected. Fragmented DNA was detected after the increase and nuclear localisation of p53.

5.3.4.3 Gene expression

Differences in gene expression between treated and non-treated SH-SY5Y cells were investigated by means of cDNA RDA after 12 hours of treatment with 10 µg/ml of juniper berry extract (IV). The time period was selected to demonstrate the gene expression before the apoptotic events indicated by DNA fragmentation. After 12 hours of treatment, the amount of p53 began to increase, metabolic activity decreased, though the cell membrane was intact. Fragmented DNA indicating apoptotic cell death was not detected until 24 hours after treatment. In total, 21 differentially expressed genes related to various functions were detected, including cell death and survival, cell cycle, cellular stress, cell shape, motility and polarity, protein synthesis, Ca^{2+} signalling, and protein-protein interactions. Several predicted proteins of differentially expressed genes were related to events in cellular stress conditions: HSPA5 (BiP), CALM2 (calmodulin), and YKT6 (a component of SNARE) regulate ER stress; MORF4L1 (MRG15) responds to DNA damage; RPL6 and RPLP0 act on ribosomal stress; and Rac1 mediates cell survival under cellular stress. All of these events may be responsible for the detected activation of p53. In addition, genes directly inhibiting, activating, or interacting with p53 were detected (Figure 9). For example, RPL6 inhibits Mdm2 which is the main inactivator of p53, and STRADA and RAC1 activate LKB1 which is an autophagy-related transcriptional target of p53. Other proteins promoting autophagy and apoptosis are, for example, MORF4L1, YKT6, and CSDE1.

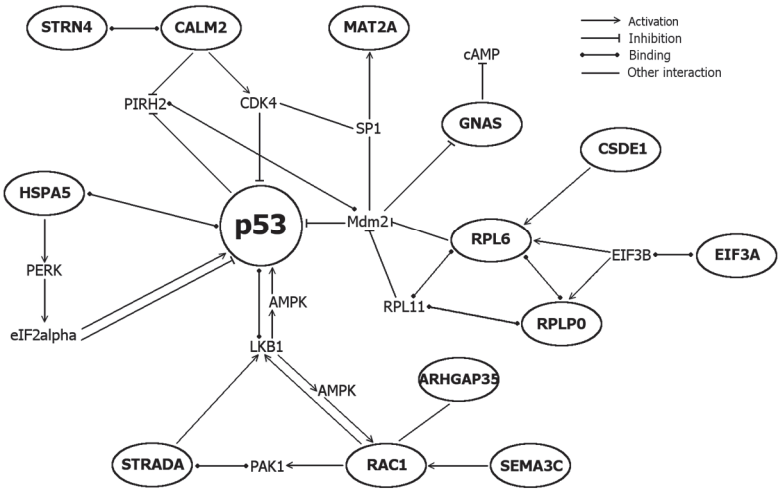

Figure 9. Interactions of differentially expressed genes and p53 (in circles). Interactions were assessed for predicted proteins encoded in the STRING 9.1 database, accompanied with a literature search.

5.3.4.4 Phenolic composition and treatment concentrations

The main plant phenolics of juniper berry extract were derivatives of quercetin, apigenin, isoscutellarein, and hypolaetin (IV). They comprised 80% of the identified phenolic compounds of juniper extract. Treatment concentrations for single compounds varied from 2.53 to 38.10 nM (Table 6).

Table 6. Phenolic composition analysis of juniper berry extract. Plant phenolics are presented in order of quantity.

PLANT PHENOLIC	CLASS	SUB-CLASS	AMOUNT IN EXTRACT µg/g	TREATMENT CONCENTRATIONS nM (ng/ml)
Quercetin	Flavonoid	Flavonol	2068	37.95 (20.68)
Apigenin	Flavonoid	Flavone	1646	38.10 (16.46)
Isoscutellarein	Flavonoid	Flavone	1225	28.62 (12.25)
Hypolaetin	Flavonoid	Flavone	1192	27.01 (12.02)
Procatechuic acid	Phenolic acid	Dihydrobenzoic acid	412	26.75 (4.12)
Catechin	Flavonoid	Flavanol	406	14.00 (4.06)
Kaempferol	Flavonoid	Flavonol	250	5.58 (2.50)
Amentoflavone	Flavonoid	Biflavonoid	167	3.10 (1.67)
Gossypetin	Flavonoid	Flavonol	155	2.53 (1.55)
Luteolin	Flavonoid	Flavone	109	2.61 (1.09)
Rosmarinic acid	Phenolic acid	Caffeic-acid ester	103	2.86 (1.03)

6 DISCUSSION

Plants are an interesting and affordable natural source of drug candidates, and their main secondary metabolites, phenols and extracts rich in phenols, have been intensively studied for their mechanisms of action on cell-death and related cell-signalling pathways (Brglez-Mojzer et al. 2016, Anantharaju et al. 2016). Plant phenolics and extracts are known for their antioxidant activity and their direct modulatory and low-affinity interference with proteins mediating cell signalling in cell death. The potential synergistic and additive effects, with modulatory actions, of molecules on their cellular targets provide an approach for the prevention and treatment of complex diseases such as cancer.

In this study, the plant phenolics curcumin, resveratrol, and quercetin, and plant extracts from basil, juniper, laurel, lemon balm, parsley, and Siberian pine were investigated for their cytotoxic and cell-signalling effects on cell death. The main focus was on extracts and their potential additive or synergistic effects. Human SH-SY5Y neuroblastoma and A375 melanoma cells were considered as cancerous cell models, and monkey CV1-P fibroblast cells as a non-cancerous cell model. The main objective of the cells was to represent cancer generally, rather than to study specific cancer types. Neuroblastoma cells were selected due to their proven sensitivity in cytotoxicity studies (Puttonen et al. 2008), and because they contain functional wild-type p53, which is able to act as a transcription factor (Tweddle et al. 2001). A375 melanoma cells contain constantly elevated levels of transcription factor NF-κB (Munshi et al. 2004) and wild-type p53 (Min et al. 2005). These cells were selected in order to study both the apoptotic cell-signalling and anti-inflammatory effects of plant material. Non-cancerous fibroblast cells were used as control cells in order to optimise test methods, and in order to be able to compare effects on cancerous and noncancerous cells. Before testing the plant extracts, the repeatability and reliability of the methodology and cell models were optimised using AraC and the well-known plant

phenolics, curcumin and resveratrol. The results using AraC were comparable to known effects (Manakova et al. 2003, Puttonen et al. 2008).

Cytotoxicity was demonstrated by the decrease of the metabolic activity of cells with an MTT test, and membrane integrity with an LDH test, which were used to distinguish regulated (e.g. apoptosis) and accidental cell death (necrosis). The leakage of LDH from cells has been proposed as a marker of necrotic or accidental cell death (Chan, Moriwaki and De Rosa 2013). However, the distinction is not definitive without further experiments due to recent findings that show that some necrotic-death types are regulated (Chan, Luz and Moriwaki 2014). The effects of plant material on cell signalling were investigated by following three proteins, p53, Bcl-2, and p65. The tumour suppressor p53 is the one of the key regulators of cell-cycle arrest and apoptosis. It is capable of transcriptional activity in the nucleus, of modulatory interactions with proteins in the cytoplasm, and detects different signals of cellular stress (Carvajal and Manfredi 2013). Bcl-2 is an anti-apoptotic protein acting with other Bcl-2-family proteins in mitochondrial and death receptor-mediated pathways of apoptosis (Czabotar et al. 2014). Protein p65 is an active sub-unit of transcription factor NF-κB with DNA binding activity. It is located in the cytoplasm and translocates to the nucleus when activated (Scott et al. 1993).

The antioxidant and pro-oxidant activities of plant extracts have been reported in numerous studies (e.g. Dai and Mumper 2010). Aqueous (Aq) plant extracts from basil, juniper berries, laurel, and parsley were selected for this study due to their varying phenolic content and antioxidant activity, as measured by Hinneburg, Dorman and Hiltunen (2006). Methanol (MeOH) extract of parsley, acetone (Ac) extract of Siberian pine, and ethanol (EtOH) extract of lemon balm were investigated in parallel with antioxidant studies (II, III, Dastmalchi et al. 2008). The estimated phenolic content of extracts studied, from the highest to slowest concentration, were detected in lemon balm (EtOH) > Siberian

pine (Ac) > basil (Aq) > laurel (Aq) > parsley (Aq) > juniper (Aq) > parsley (MeOH). When comparing antioxidant activities measured with DPPH free -radical scavenging, extracts, from the strongest scavengers to the weakest, were lemon balm > Siberian pine > basil = laurel > juniper > parsley (MeOH) > parsley (Aq). Extracts with high total phenolic content and strong antioxidant activity induced necrotic cell death without stabilisation of p53, or p53 stabilised simultaneously with massive cell death. Parsley, with low total phenolic content and weak antioxidant activity, was not relatively toxic to cells. Only juniper extract, with relatively high antioxidant activity and low phenolic content, induced the stabilisation of p53 at low treatment concentration. Results from the strong antioxidants indicate that their detected toxicity was more likely caused by disturbance of the oxidant-antioxidant equilibrium following oxidative stress or shock by phenolic-rich treatments than due to activation of cell-death signalling (Halliwell 2014). The results are in line with *in vitro* and *in vivo* observations that high concentrations of plant phenolics increase their toxicity, and may contribute to the development or progress of cancer (Sayin et al. 2014). Therefore, phenolic content and antioxidant activity can be used as an indicator of cytotoxicity, but not for the effects on cell signalling *in vitro*.

The selectivity of plant phenolics in inducing cell death in cancer cells, but not in non-cancerous cells has been reported for several phenols and phenolic-rich plant extracts. Comparison of such selective sensitivity was tested with curcumin, resveratrol, and methanol extract of parsley. Both curcumin and resveratrol are plant phenolics well-known and intensively studied for their anti-cancer effects (Sa and Das 2008; Delmas, Solary and Latruffe 2011), and both of them have been demonstrated to selectively kill cancer cells (Ravindran, Prasad and Aggarwal 2009; Goel and Aggarwal 2010; Gwak et al. 2015). In this study, curcumin showed selective toxicity against cancerous neuroblastoma cells without effects on cell viability or on p53 in non-cancerous fibroblast cells. Similar selective toxicity towards cancerous melanoma cells was found with parsley extract, with only weak effects on non-cancerous fibroblast cells. Other studies to demonstrate

selective sensitivity towards different cells are not available for parsley. In contrast to former studies, resveratrol showed cytotoxic effects towards non-cancerous fibroblast cells, but not towards cancerous neuroblastoma cells. These controversial effects may be explained by the cytoprotective effects of resveratrol demonstrated in several former studies (e.g. Juhasz et al. 2010).

The mechanisms of action behind the observed effects discussed above were evaluated by means of metabolic activity, membrane integrity, stabilisation of p53, and activation of caspase 3. Selective toxicity of curcumin towards cancerous cells was observed by means of the stabilisation of p53 and a decrease of metabolic activity. In the literature there are several studies available that demonstrate similar effects, for example, it has shown inhibition of NF-κB signalling, increase of p53 activity, and activation of ER stress (Vallianou et al. 2015). The results of this study are in line with former studies. Resveratrol has two optical isomers. The biologically active and more stable isomer trans-resveratrol was investigated in this study (Tsai, Ho and Chen 2017). Although its anti-cancer effects are well-defined in the literature (Delmas, Solary and Latruffe 2011), resveratrol had no effects on neuroblastoma. The results with the strong antioxidants – basil, laurel, lemon balm and Siberian pine extracts – showed cytotoxicity but not the involvement of p53. For parsley, two different extracts were tested. An aqueous extract showed no effect on neuroblastoma cells, but the methanol extract decreased the metabolic activity of melanoma cells without the activation of caspase 3. Similar effects were demonstrated by Fashori et al. (2014) with an extract from parsley seed, which decreased the metabolic activity of carcinoma cells (Farshori et al. 2014). The choice of extraction method should be considered when investigating plants. Most of the extracts in this study were prepared with water, which mimics the traditional use of plants. That method may not be as efficient in obtaining phenolic-rich extracts but it is safer in use, as was demonstrated with parsley. The results with extracts, except with the juniper berry and parsley extracts, indicate necrotic cell death.

Due to the interesting effects of juniper berry extract on p53 and cell viability at low treatment concentrations, the extract was selected for further study. To better understand the mechanisms of action of juniper, its phenolic composition was analysed. Fifteen plant phenolics were identified; twelve flavonoids, two phenolic acids and one biflavonoid. Quercetin glycosides represented 26.7%, apigenin-7-O-glucoside 21.3%, isoscutellarein glycosides 15.8%, and hypolaetin glycosides 15.4% of the identified phenolic content. Other water soluble chemicals possibly present in the extract are cumarins, other secondary metabolites, and carbohydrates. Volatile oils were removed from the extract, and their absence was verified. Similar content was found for juniper by Innocenti et al. (2007) in berries collected from Italy.

Further studies of the SH-SY5Y neuroblastoma cell model revealed that juniper treatment induces the translocation of p53 to the nucleus, accompanied by the expression of ER stress-related genes, including CALM2 (protein calmodulin), YKT6 (protein Ykt6) and HSPA5 (protein BiP), among other genes related to cell cycle, Ca^{2+} signalling, and cell death. A time-response assay revealed that the observed events lead to DNA fragmentation, which is one of the hallmarks of apoptosis. Differentially expressed genes are in line with other observed effects of p53. The association of p53 and expressed genes were found for 13 of them. These results indicate that juniper extract induces apoptosis by means of p53 and ER stress in SH-SY5Y neuroblastoma cells. This is a novel finding for juniper berry extract. In the literature, there are few studies on juniper and most of them are performed with juniper volatile oils. However, recently Lange and co-workers (2015) observed similar effects in neuroblastoma cells with a methanol extract of *Juniperus oblonga* M. Bieb. leaves, flowers, roots and stems. The extracts decreased cell viability, and increased intracellular Ca^{2+}, mitochondrial membrane permeability, and apoptosis via cleavage of caspase 3 and PARP. The authors proposed that the observed effect may be related to ER – mitochondria calcium signalling.

Correspondences between the results in this study and those of Lange et al. (2015) could be explained by similar phenolic contents of juniper extracts (Pisarev et al. 2011). Quercetin and apigenin are the main phenols in juniper berry extract, and both have shown effects on apoptotic cell signalling in several cancerous cell lines (Gibellini et al. 2011; Shukla and Gupta 2010). In this study, quercetin showed apoptotic effects by increasing caspase 3 activity and translocation of p53 to the nucleus in A375 melanoma cells, but it did not show any anti-inflammatory effects on NF-κB p65. He et al. (2016) have shown that quercetin induces apoptosis by activating ER stress followed by the up-regulation of pro-apoptotic Bax and release of cytochrome c, down-regulation of anti-apoptotic Bcl-2, and caspase activation. In the study by Yang et al. (2015), quercetin enhanced the cytotoxicity of the chemotherapeutic agent cisplatin by inducing ER stress in human ovarian carcinoma cells and mouse ovarian tumour xenografts. The mechanisms of apigenin for inducing apoptosis in neuroblastoma cells include the induction of p53, caspase activity, PARP cleavage, an increase in the levels of Ca^{2+} and the Bax:Bcl-2 ratio, and the release of cytochrome c from mitochondria (Das, Banik and Ray 2006; Torkin et al. 2005). Apigenin has been shown to stabilise both wild-type and mutated p53, and to induce the expression of p21 in various cancerous cell lines (McVean et al. 2000, Takagaki et al. 2005). The detected involvement of ER stress in quercetin-induced apoptosis and the involvement of p53 in apigenin- and quercetin-induced apoptosis support the observations regarding juniper berry extract in this study, although the apoptotic and cytotoxic effects of juniper cannot be explained by the activity of a single phenolic compound due to their low concentrations in the extract. The concentrations of single phenolics in the treatments were at nanomolar level, while the effective concentrations of apigenin and quercetin are at micromolar level. The involvement of synergistic or additive effects may explain the observed effects of juniper berry extract. In order to confirm an action of synergy, different methodological approaches have been developed, including a combination index (Chou 2006, Zhou et al. 2016).

There are several challenges to using plant extracts in drug development partly due to their unknown and variable content. Environmental growing conditions with biotic (pests and pathogens) and abiotic (temperature, light, dryness) stresses, and extract preparation methods affect the content of plant secondary metabolites, including phenolics (Dixon and Paiva 1995, De Abreu and Mazzafera 2005). For example, Tavares and co-workers (2013) demonstrated that the highest phenolic content was measured during the slowest growing period in four different juniper species. Speed of growth did not affect the qualitative content of phenolics, which changed under different stress conditions. Product preparation processes may cause the loss of phenolic compounds, or qualitative and quantitative variation (Dai and Mumper 2010). This variability may not be remarkable, as seen in the different extraction methods applied to juniper, but it may affect the biological activity of the product, as demonstrated in this study with the two parsley extracts. In order to successfully use plant extracts, growing conditions should be addressed, and standardisation of extracts is crucial in order to verify the quality of the product. Bioavailability and the metabolism of plant phenolics might change the predicted effects in *in vivo* conditions (Hu et al. 2010). As a result of poor bioavailability and extensive modifications by intestinal, microflora and liver enzymes, phenolics in target organs may not correspond to the phenolics ingested or tested in *in vitro* assays.

Plant phenolics possess a health-beneficial role when ingested as part of a diet. In order to find plant-derived drug candidates from edible plants for complex diseases such as cancer, an approach that investigates synergistic or additive effects and non-specific modulatory mechanisms of actions, rather than finding single molecules for single targets, should be considered. The benefits of such an approach are lower toxicity and possibility of reducing the development of resistance in cells.

7 CONCLUSIONS AND FUTURE PROSPECTS

In this study, cancerous melanoma and neuroblastoma and non-cancerous fibroblast cell models were used in order to investigate cytotoxicity and the apoptotic effects of plant phenolics and extracts with known total phenolic content and antioxidant activities. Cytotoxicity and cell viability (MTT and LDH tests), the proteins p53, Bcl-2, and NF-κB (Western blot), caspase 3 activity, DNA fragmentation, and differences in gene expression were investigated.

- Assessment of the predictability of cytotoxicity and cell-death signalling in relation to the total phenolic content and antioxidant activity in *in vitro* cell models demonstrated that these properties can be used as predictors of cytotoxicity, but not for cell-death signalling. The selection of plant extracts as drug candidates cannot be based only on the measured antioxidant activity, but also on their specific phenolic content and traditional uses. More detailed quantitative analyses for the prediction of cytotoxicity are suggested for future studies.

- Comparison of the selective sensitivity of curcumin, resveratrol, and parsley extract on cancerous and non-cancerous cells demonstrated that cancerous cells are more sensitive to curcumin and parsley extract than are non-cancerous cells. Similar selective sensitivity was not observed with resveratrol. The results for curcumin and resveratrol are in line with observed selectivity in the literature. Comparable studies for parsley are not available. The results confirm that plant phenolics may be selective towards cancerous cells, providing a beneficial feature for the prevention or treatment of cancer.

- Evaluation of plant phenolics and extracts for their cytotoxicity and cancer-related cell signalling demonstrated that extracts from basil, laurel, lemon balm, Siberian pine, and parsley had no effects on cell

signalling and p53, and that the necrotic cell death detected was most probably a consequence of cytotoxicity triggered by oxidative stress, in turn triggered by high concentrations of phenols. Only juniper berry extract induced the stabilisation of p53 without changes in membrane integrity. The detected cytotoxic effects may be of interest in relation to recent knowledge regarding the regulated mechanisms of necrosis.

- Juniper berry extract was selected for further testing for its effects to stabilise p53. Translocation of p53 in the nucleus, accompanied by the expression of genes related to ER stress and cell death, and DNA fragmentation after the induction of p53, resulted in apoptotic cell death induced by ER stress. The observed effects cannot be explained by any single identified plant phenolic due to the low concentrations of phenols, a circumstance which supports the argument for potential synergism. These results support the use of plant extracts for their synergistic effects in drug development, and foreground juniper berries as a potential drug candidate for the prevention or treatment of cancer.

As regards further steps, due to the enormous *in vivo* biotransformation of phenolic compounds, *in vitro* testing of metabolites of juniper berry extract is recommended. Depending on the results, *in vivo* testing in animal models may be necessary in order to confirm potential activity. Standardisation of the extract is recommended, as is confirmation of possible synergistic effects, using quantitative methods such as the recently introduced combination-index test, in advance of further testing.

ACKNOWLEDGEMENTS

This work was carried out at the Divisions of Pharmaceutical Biosciences and Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki, Finland, and at the University of Tartu, Estonia. It was financially supported by the Graduate School in Pharmaceutical Research, and the Finnish Cultural Foundation.

My greatest gratitude goes to my supervisors Professor Atso Raasmaja and Professor Raimo Hiltunen (deceased 2014). I would like to especially thank Atso for his endless patience and sincere trust in this work and me, and all the hours he has spent to support me with both bigger and smaller steps to get this work finalised. Unfortunately Raimo could not see the finalisation of this work, but I know that his spirit and passion for medicinal plants will continue to live through this work and several others.

I would also like to express my warmest gratitude to Professor Sulev Kõks for the opportunity to work in his laboratory, and his brilliant comments on the tricky analyses of gene expression studies. Professor Heikki Vuorela is acknowledged for his support, especially with the last steps of this work.

All co-authors are warmly acknowledged for their valuable contribution to the practical and theoretical work, and interesting scientific and general discussions.

My sincere gratitude goes to Professor Matti Viluksela and Associate Professor Anna Jäger for their critical and insightful review of my thesis.

I am most grateful to my former and current colleagues for creating the most inspiring and supporting atmosphere for working. Special thanks go to Docent Carina Tikkanen-Kaukanen, who introduced me to the treasures of nature in drug research, MSc Johanna Peltola-Thies for all the encouragement and support to finalise this work, and to my closest colleagues Anna Galkin, Päivi Järvinen, Nahed El-Najjar, and Damien

Dorman, with whom I had an opportunity to share both happy and challenging moments, laughs and tears during my ”laboratory years”.

My warmest thanks go to my parents, family and friends. Especially my late father Arto and grandmother Aino, thank you for always believing in me. Your heritage will live in my heart for the rest of my life. My ”mother-in-love”, Pirkko: I will always be grateful for your special support. Mikko, Outi, Riikka, Santeri, Satu, Teija, Tuukka and Virpi: thank you for your unconditional friendship and support.

The last but not least thanks go to my dear husband Perttu for his love and understanding which carried me through the challenging moments finishing this work.

Helsinki, October 2017

Tiina

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Helsinki 2017 ISSN 2342-3161 ISBN 978-951-51-3799-9